PHENOTYPIC AND FUNCTIONAL CHARACTERIZATION OF EQUINE MONOCYTE-DERIVED DENDRITIC CELLS

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Derek John Cavatorta

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Derek John Cavatorta, D.V.M., Ph.D.

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This dissertation was motivated by the desire to further our understanding of the immune response to vaccination and with the hope of promoting the development of improved vaccine strategies. These studies have focused on the equine dendritic cell (DC) because of the important role this cell plays in initiating the immune response; DCs are unique in their ability to optimally sensitize naïve T cells and are capable of promoting the development of a range of effector T cell phenotypes. In particular, the phenotypic and functional characteristics of the equine monocyte-derived DC were characterized.

Monocyte-derived DCs and macrophages were stimulated with UV-inactivated *Escherichia coli* (*E. coli*) and monitored for cell surface marker expression, cytokine production, and endocytic capacity. The resulting alterations in DC activation state were characterized, and the differences between DCs and macrophages were further defined. These findings contribute to our knowledge of equine DCs and demonstrate that, although non-stimulated DCs consist of a mixed population of mature and immature cells, DC maturation can be measured following induction by bacterial stimuli.

A method for characterizing DC function was also developed. Relatively pure populations of equine monocyte-derived DCs were co-cultured with autologous, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained peripheral blood T cells. Multi-

color flow cytometry was used to measure antigen-specific T cell proliferation, surface-marker expression, and cytokine production. The DC-induced T cell response was characterized in response to both self-antigen and vaccine antigen. These experiments confirmed the potent antigen-presenting capabilities of equine DCs, which validates their immunotherapeutic potential and supports their use as a cellular vaccine adjuvant. This system also permitted the use of DCs to study fundamental immunological processes *in vitro*. These findings contribute to our knowledge of the equine immune system and demonstrate the value of DCs as a research tool.

A lymphoscintigraphic procedure for identifying the vaccine-draining lymph node in the horse was also established. This technique will facilitate the study of the anti-vaccine immune response and encourage the development of improved vaccination strategies, such as those using DC-based adjuvants.

BIOGRAPHICAL SKETCH

Derek Cavatorta was born the left-handed, identical twin son of John and Elaine Cavatorta on their third wedding anniversary—September 3rd, 1981—in Boston, Massachusetts. He and his brother, Jason, were born nearly 10 weeks prematurely and weighed just 3 pounds apiece. His younger brother, Marc, was born in 1984 and his sister, Ali, followed in 1985. Derek was raised in the small, coastal town of Rowley and developed a strong affinity for science and nature during a childhood with much time spent exploring the creeks and beaches of Ipswich Bay and its tributaries.

Derek attended the Pine Grove Elementary School and Triton Regional High School, where his interest in veterinary medicine was encouraged by a profoundly influential science teacher and wildlife rehabilitator, Mr. David Taylor. In Dave's Natural Science course, students cared for injured native wildlife, developed independent research projects, and assisted in the necropsy and skeletal recovery of deceased whales and dolphins. Derek's love of science was also fostered by his physics teacher, the late Mr. Christopher Haven, who remains a true inspiration. Derek was the valedictorian of his class and graduated in the spring of 2000.

Derek earned his B.S. degree in Animal Science from the University of Massachusetts, Amherst. It was at UMass that Derek developed an interest in working with large animals. He fondly recalls the many school breaks spent working with horses and cattle as a veterinary technician for Dr. Robert Orcutt and Dr. Helen Noble at SRH Veterinary Services in Ipswich, MA. He developed an interest in research during a summer internship in which he studied North Atlantic Right Whale fishing gear entanglement at the Woods Hole Oceanographic Institute on Cape Cod with Dr. Michael Moore. Derek's decision to pursue graduate training

was solidified after taking an exceptional immunology course taught by Dr. Barbara Osborne. He graduated *summa cum laude* in 2004.

Derek began the dual DVM/PhD degree program at the Cornell University College of Veterinary Medicine in Ithaca, New York, in the fall of 2004. He remains extremely grateful for his acceptance into this program and considers it the opportunity of a lifetime. Derek met his girlfriend, Dr. Lauren Sawchyn, in the vet school library (appropriately) during his second year at Cornell, and she has been stuck with him ever since. He completed his veterinary degree in 2010.

Derek has loved living in Ithaca for the past 8 years. A lifelong running enthusiast, he has particularly enjoyed the hundreds of Saturday mornings spent running through the gorges of Ithaca with Ian Golden and company. He completed his first ultramarathon in September of 2011, just after his 30th birthday, to convince himself that obtaining two doctoral degrees hadn't completely sapped his youthful vitality. After graduation, Derek and Lauren will move to Massachusetts with their pets, Rosey and Joppa, where Derek will complete a large animal internship at SRH Veterinary Services.

To my father, for teaching me to value hard work.

To my mother, for teaching me to value life.

To Lauren, for helping me to balance the two.

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I am eternally indebted to many people for all of their help over the past 8 years; the challenges associated with completing a combined DVM/PhD program are obvious, and the overwhelming support I have received has been crucial to my success.

First and foremost, I would like to thank my mentor, Dr. Julia Felippe. In particular, I would like to thank Julia for creating a laboratory environment that afforded me the opportunity to perform sophisticated immunology research in a veterinary species—such labs are not common. I would also like to thank Julia for providing me with an abundance of academic freedom. Several of the projects I worked on were tangential to the major laboratory objectives and had no specific funding, but Julia remained supportive throughout. Finally, I would like to thank Julia for her understanding of the constraints on my time during my veterinary training. I know how frustrating that must have been, and I appreciate her patience.

I would like to thank the other members of the Felippe lab for all of their help. I thank Mary Beth Matychak for her friendship, for sharing her vast knowledge of innumerable laboratory techniques, and for her expert cooking instruction—I will miss her carrot cake nearly as much as I miss her kindness and personal support. I thank Dr. Rebecca Tallmadge Ingram for her unending patience and for many helpful discussions. Becky has been a great resource and a huge help with planning experiments and interpreting data. I also thank Jenny Battista, Michelle Hilton, and Dr. Ute Schwaab for all of their help and for creating a friendly environment in the lab.

I would like to acknowledge the members of my graduate committee—Dr. Ted Clark, Dr. Dave Holowka, and Dr. Collin Parrish—as well as my external examiner, Dr. Julia Kydd, for all

of their guidance. I wish to express my thanks to the past and present members of the dual degree committee, particularly Dr. Linda Nowak, for providing me with this amazing opportunity. I thank Dr. Douglas McGregor and Dr. John Parker for their advice and grant support. I am extremely grateful for all of the administrative support provided by Ms. Janna Lamey—her assistance is crucial to the success of the whole department. I would also like to thank Dr. Hollis Erb for her statistical help, Dr. James Casey and Dr. Lisa Fortier for allowing me to rotate through their laboratories, and Dr. Joe Wakshlag and Dr. Fran Kallfelz for their sage career advice.

Finally, I thank my family and friends for all of their love and encouragement. I especially want to thank my girlfriend, Dr. Lauren Sawchyn, for her interminable support and for the beautiful illustrations featured in this dissertation.

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INTRODUCTION

The equine immune response to vaccination represents an important interface between veterinary medicine and immunology; administering vaccinations to protect patients against a variety of pathogens is an essential role of the equine veterinary practitioner, and a fundamental comprehension of the underlying immunological processes is necessary to design optimal vaccine strategies. In the horse, as in other species, this strategy varies by patient according to the animal's age, pregnancy status, immune status, and risk of disease exposure. However, when the recommendations of the American Association of Equine Practitioners (AAEP) are followed, all horses receive many vaccinations throughout their lives (Table 1.1). For example, a typical foal in the northeastern United States receives approximately 15 vaccinations before it is a year old (some of which are combined into a single injection). It is important to bear in mind that these vaccines are administered at some expense to the horse owner and are not without risk to the patient (Mair. 1988; Kemp-Symonds et al., 2007; Timoney. 2007). Furthermore, there are commercially available equine vaccines for which there is little to no evidence of clinical efficacy, and there are no vaccines available for several prevalent equine diseases (Barquero et al., 2007).

This dissertation was motivated by the desire to further our understanding of the immune response to vaccination and with the hope of promoting the development of improved vaccine strategies. In particular, I have focused my studies on the equine dendritic cell (DC) because of the important role this cell plays in generating an immune response to vaccination. The equine DC may also be useful as a tool to study the immune system *in vitro* and therapeutically manipulate the immune system *in vivo*. A brief, general description of vaccinology will ensue, followed by a detailed discussion of pertinent immunological principles.

Table 1.1. Sample vaccination schedule for a horse in the northeastern U.S.

Pathogen	Type of Vaccine	Foal Series	Booster Frequency
Tetanus	Inactivated toxoid	3 doses	Annual
EEE/WEE	Inactivated	3 doses	Annual
WNV	Inactivated	3 doses	Annual
	Recombinant	3 doses	Annual
	Inactivated chimera	3 doses	Annual
Rabies	Inactivated	2 doses	Annual
EHV-1	Inactivated	3 doses	Biannual
	Modified live	3 doses	Biannual
Influenza	Inactivated	3 doses	Biannual
	Modified live	2 doses	Biannual
	Recombinant	2 doses	Biannual
Strangles	Killed	3 doses	Annual
	Modified live	2 doses	Annual

1.1. Principles of Vaccinology

At a basic level, vaccination involves administering a non-pathogenic preparation of an infectious pathogenic organism to the host with the intention of stimulating both short-term and long-lasting immunity. Antigen is delivered to the peripheral tissues in the form of a killed pathogen, a pathogen subunit, a modified live pathogen, or a more complicated delivery system such as a DNA or vectored vaccine (El Garch et al., 2008; Liu. 2010). In recent years, the use of

advanced molecular methods including reassortment, recombination, deletion mutants, codon deoptimization, and reverse genetics have also been employed in vaccine development (Plotkin. 2009). Antigen delivery may be obtained by multiple routes, including intramuscular, subcutaneous, intradermal, intranasal, oral, sublingual, and transcutaneous (Yuki and Kiyono, 2009). The form of the antigen and the method of delivery have a large effect on the nature of the immune response generated. In addition, many vaccines contain adjuvants, which further enhance and modify the vaccine response (Harandi et al., 2009).

Regardless of the antigen source or delivery method employed, the vaccine antigen must be processed and presented by an antigen-presenting cell (APC) in order to generate an efficient immune response. DCs are potent APCs that are present in virtually every tissue of the body and possess the unique ability to stimulate naïve T cells (Sato and Fujita, 2007). They were first identified in the adherent fraction of cells from mouse peripheral lymphoid organs by Ralph Steinman and characterized based on their distinct morphological features (Steinman and Cohn, 1973). In the intervening years, we have learned that DCs are the master regulator of the immune system and are able to direct the development of a wide range of immune responses to antigen. A detailed understanding of DC biology, including maturation, migration, ability to stimulate T cells, and division into subsets is crucial to enhancing our knowledge of the immune system and to developing effective vaccination strategies (Steinman and Hemmi, 2006).

1.2. Dendritic Cell Maturation

Immature migratory DCs reside in the periphery, particularly the skin and mucosal tissues, where they are strategically located to detect invading pathogens or tissue damage. Such DCs actively sample the environment via macropinocytosis of soluble antigens and receptor-

mediated phagocytosis of particulate antigens (Sallusto et al., 1995). In the absence of infection or inflammation, the majority of the antigens encountered by immature DCs in the periphery are self-antigens. Fortunately, immature DCs are unable to activate any potentially autoreactive T cells due to low expression of major histocompatibility complex (MHC) and co-stimulatory molecules. In addition, immature DCs are unlikely to encounter T cells in the peripheral tissues since T cells are primarily located in the bloodstream and lymphoid tissues in the steady state. Therefore, the immature phenotype and anatomic isolation of immature DCs allows them to remain vigilant sentinels for invading pathogens without inducing immune-mediated disease.

DCs express an array of evolutionarily-conserved, germline-encoded receptors involved in detecting "danger signals" that indicate a threat to the host. These receptors include pattern recognition receptors (PRRs) that bind to pathogen-associated molecular patterns (PAMPs) and other receptors that detect host-derived signals such as inflammatory cytokines (including IL-1, TNF-α, and IL-6) and alarmins released following tissue injury (also referred to as damage-associated molecular patterns or DAMPs) (Jonuleit et al., 1997; Schreibelt et al., 2010). The Toll-like receptors (TLRs) represent a large, well-characterized family of PRRs that recognize molecules produced by many microbes. For example, TLR2 recognizes multiple components of Gram+ bacterial cell walls; TLR3 is activated by viral double-stranded RNA; TLR4 binds to LPS on Gram- bacteria; TLR5 is activated by flagellin on flagellated bacteria; and TLR9 recognizes unmethlyated CpG motifs (Guermonprez et al., 2002). TLRs are found on the cell surface (TLRs 1, 2, 4, 5, 6, 10, and 11) or within endosomal compartments (TLRs 3, 7, 8, and 9). Upon ligand binding, TLR signaling occurs through MyD88-dependent and TRIF-dependent pathways that activate transcription factors such as NFκB that mediate DC activity. DCs express many other PRRs, including cytoplasmic NOD-like receptors that recognize bacterial products; viral

receptors; C-type lectin receptors, including CD206 and DEC-205, which recognize bacterial carbohydrates; Fc receptors that bind opsonized pathogens or immune complexes; and receptors for heat shock proteins released by necrotic cells (van Vliet et al., 2007).

Different DC subsets express different PRRs and different PRRs induce different signaling pathways so that the DC response to infection is tailored to the PAMP signature of a given pathogen. For example, TLR3 and TLR4 act synergistically with TLR7, TLR8, and TLR9 to induce production of IL-12 and IL-23 by human DCs (Napolitani et al., 2005). PAMP-induced DC cytokine production has a profound impact on the nature and efficacy of the developing immune response (section 1.5. T Cell Stimulation).

Recognition of danger signals initiates DC maturation, a metamorphosis that represents a transition from a cell specialized in pathogen detection and antigen uptake to one devoted to antigen presentation and initiation of the adaptive immune response. At the completion of this developmental program, the DC becomes an extremely potent APC and is unique in its ability to optimally sensitize naïve T cells. DC maturation involves reduction of endocytic activity, upregulation of co-stimulatory molecules such as CD40, CD80, and CD86, enhanced expression of MHC-peptide complexes, production of pro-inflammatory cytokines, and acquisition of cellular mobility (Schuurhuis et al., 2006).

The mature DC migrates from the periphery to regional, organized lymphoid tissues, a highly complicated and coordinated process that is crucial to the development of a successful immune response (Figure 1.1A). For example, Langerhans cells (LCs) are a DC subset that resides in the suprabasal layer of the epidermis under steady state conditions. LC maturation induces upregulation of the chemokine receptor-7 (CCR7), which exclusively binds to the chemokines CCL19 and CCL21 (Förster et al., 2008). These chemokines are produced by

fibroblastic reticular cells in the T cell-rich lymph node paracortex and diffuse through the lymphatic vessels to generate a chemical gradient. CCR7 signaling in the mature LC initiates migration along this gradient, which involves altering cytoskeletal organization, breaking down bonds with neighboring keratinocytes, digesting through the basement membrane, traversing the dermal connective tissue, intravasating into lymphatic vessels, and traveling down the afferent lymphatics to the draining lymph node (Romani et al., 2001). The migratory DC localizes to the lymph node paracortex, where it is strategically situated to present peptides processed from antigens encountered in the periphery to antigen-specific naïve T cells. Binding of CD40L (CD154) and other receptors on the surface of the T cell initiates signaling pathways that complete the DC maturation process (O'Sullivan and Thomas, 2003). In turn, the DC provides signals that induce T cell expansion and effector differentiation (section 1.5. T Cell Stimulation). In this way, DC maturation serves as the critical link between innate and adaptive immunity (Steinman. 2011). This method of motocrine signal transduction is extremely rare and is made possible by the elegant interplay between the immune system and the regional lymphatic anatomy (Figure 1.1) (Zinkernagel et al., 1997).

1.3. Lymph Formation and Flow

The lymphatic system serves an essential immunological function by allowing the transport of antigens, chemokines, and leukocytes throughout the body (Figure 1.1). A detailed understanding of the generation, filtration, and drainage of lymph is important when understanding the role DCs play in the immune response to vaccination (Swartz et al., 2008).

A continuous flow of fluid into the interstitium occurs as plasma leaks out of the blood capillaries along hydrostatic and osmotic pressure gradients known as Starling's forces. The

interstitium is a mesh-like network of extracellular matrix (ECM), and pressure gradients drive the flow of fluid through the interstitium into the lymphatic capillaries (Swartz and Fleury, 2007). These vessels possess discontinuous basement membranes and weak cell-cell junctions, making them highly permeable to interstitial fluid and solutes (Schmid-Schönbein. 1990). The size of these solutes determines how readily they will be taken up by the lymphatics. In general, smaller molecules (less than 100 nm) are taken up more efficiently. However, molecules smaller than 10 nm are preferentially absorbed into the blood capillaries (Swartz. 2001). The ECM also filters macromolecules and may prevent the transport of molecules that could otherwise enter lymphatic vessels (Aukland and Reed, 1993). Other factors, such as charge and composition of molecules, have also been demonstrated to affect efficiency of lymphatic uptake (Swartz. 2001).

The endothelial cells of the lymphatic capillaries are radially tethered to the surrounding ECM by anchoring filaments (Leak and Burke, 1968). As the interstitium swells with accumulated fluid or the ECM is strained by skeletal motion or arterial pulsations, the anchoring filaments dilate the initial lymphatics. This generates negative pressure, and fluid is "pumped" into the lymphatic vessel through gaps between endothelial cells (Randolph et al., 2005). Once the fluid enters the lymphatic vessels, it is termed lymph. The lymph is channeled through a system of converging and enlarging lymphatic vessels in the following order: lymphatic capillaries (also termed initial or terminal lymphatics), collecting vessels, lymph nodes, lymphatic trunks, and lymphatic ducts. In contrast to the lymphatic capillaries, collecting vessels are not tethered by anchoring filaments. Collecting vessels possess smooth muscle fibers which contract and drive the flow of lymph forward through the lymphatics. Retrograde flow of lymph is prevented by one-way valves in the vessel lumen (Aukland and Reed, 1993). Therefore, the flow of fluid and solutes through the lymphatics is an active process and not simply a

consequence of passive diffusion. The lymphatic ducts return lymph fluid to the venous bloodstream at the venous angle, cranial to the heart.

Lymph nodes are situated along the lymphatic vessels to act as filters that sample the lymph before it reaches the blood. Lymph fluid from the afferent lymphatics enters the lymph node subcapsular sinus and is diverted around the periphery of the node to the medullary sinus before exiting the node via the efferent lymphatics. In this way, the lymph fluid is sequestered from the nodal lymphocyte compartments. However, a series of conduits lined by fibroblastic reticular cells (FRCs) channels some lymph from the subcapsular sinus to the high endothelial venules (HEVs) in the T cell-rich paracortex (Abbas et al., 2010). This allows for the delivery of lymph-borne chemokines from inflamed tissues to HEVs and subsequent recruitment of leukocytes to the node (Gretz et al., 2000). In addition, soluble antigens are taken up by lymph node-resident DCs, which are closely associated with the FRCs of the conduit system (Figure 1.1B).

Figure 1.1. The relationship between dendritic cell maturation and regional lymphatic anatomy. (A) Immature DCs reside in the peripheral tissues and function as the sentinels of the immune system. They express receptors that allow them to capture antigen and recognize microbial patterns, pro-inflammatory cytokines, and tissue damage. Upon activation by these signals, the DC undergoes a complicated maturation process that includes upregulation of CCR7. The DC can then migrate through the extracellular matrix and enter nearby lymphatic capillaries, which possess a discontinuous basement membrane. The DC follows CCL19 and CCL21 chemokine gradients down the afferent lymphatics to the sentinel lymph node. (B) The migratory DC (blue cell) enters the subcapsular sinus of the draining lymph node and crosses into the T cell-rich lymph node paracortex, where the maturation process is completed. The mature DC increases expression of MHC class I and II, upregulates co-stimulatory molecules, and increases cytokine production. In this way, the mature DC shifts from specializing in antigen capture to focus on antigen presentation and T cell activation. Alternatively, antigens can travel in the lymph to the sentinel node independently of DCs. Small, soluble antigens (<80 kDa) can enter special conduits (purple lines) that channel lymph from the subcapsular sinus to high endothelial venules (HEVs) (blue circles) in the paracortex. Lymph node-resident DCs (purple cells) line the conduits and acquire lymph-borne antigen for presentation to T cells. Naïve T cells (dark green cells) enter the lymph node from the bloodstream through HEVs in a CD62L-dependent manner. They travel through the paracortex for several hours, probing multiple DCs for presentation of their cognate antigen. If they do not encounter such a DC, the naïve T cell exits the lymph node through the efferent lymphatic in an S1P-dependent manner and returns to the bloodstream through the thoracic duct. In contrast, if the T cell does encounter a DC presenting its cognate antigen, it will remain in the lymph node, proliferate, and differentiate into effector and memory T cells (light green cells). Effector T cells exit the lymph node through the efferent lymphatics, return to the bloodstream, and mediate immunity in the peripheral tissues. (C) Depending on the DC maturation state and the local cytokine environment, activated T cells can acquire a variety of phenotypes, including: 1) IFN-γ-producing Th1 cells that are important for immunity to intracellular pathogens and tumors; 2) IL-4-, 5-, and 13-producing Th2 cells that promote antibody production, help prevent parasitic diseases, and are involved in allergic responses; 3) IL-17-producing Th17 cells that protect against extracellular bacteria; and 4) induced Tregs (iTregs), which use a variety of mechanisms (such as production of the anti-inflammatory cytokines IL-10 and TGF-β) to keep effector responses in check and prevent immune-mediated disease. Each phenotype is associated with expression of a specific transcription factor (listed in parentheses).

Figure 1.1. The relationship between dendritic cell maturation and regional lymphatic anatomy (continued).

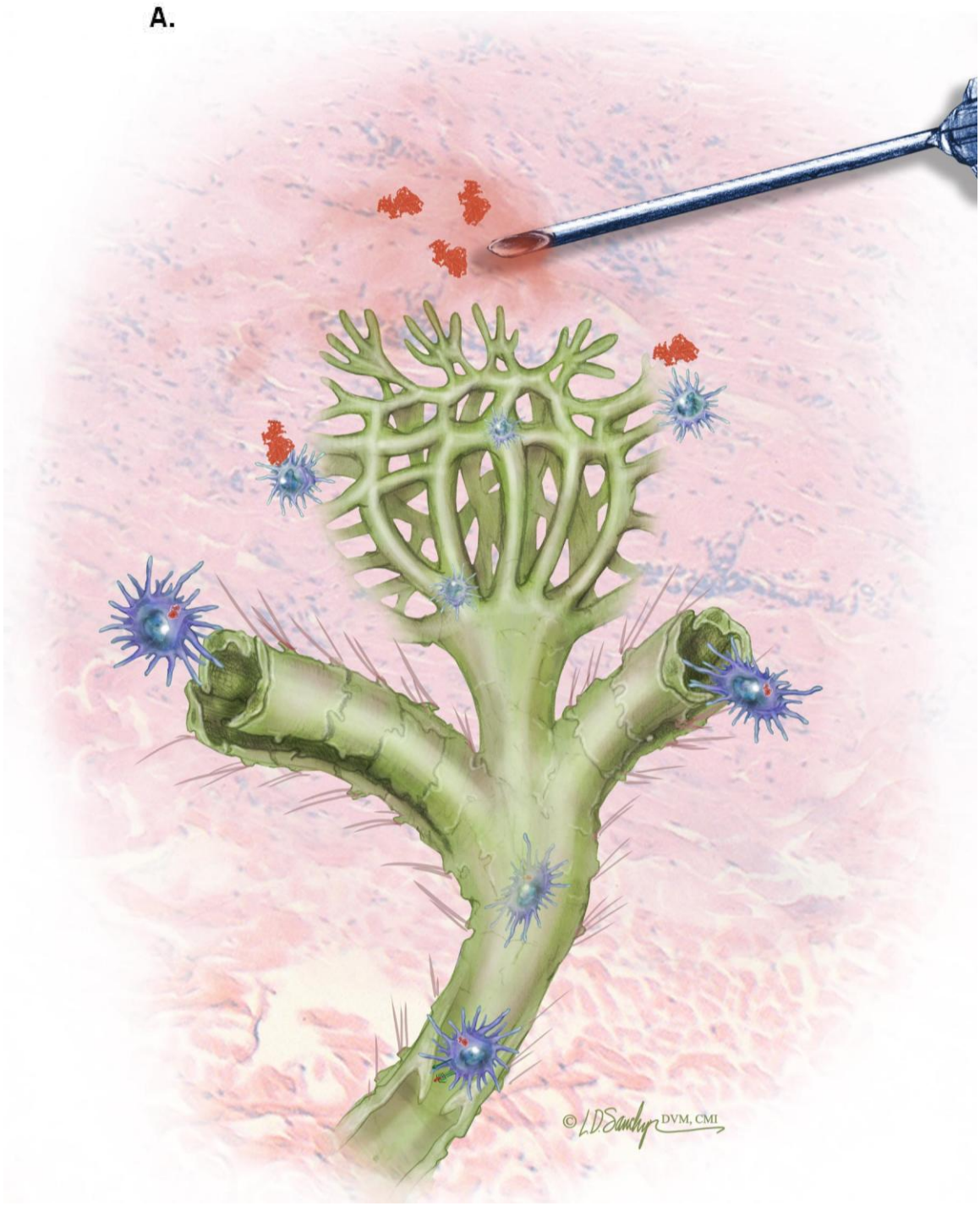


Figure 1.1. The relationship between dendritic cell maturation and regional lymphatic anatomy (continued).

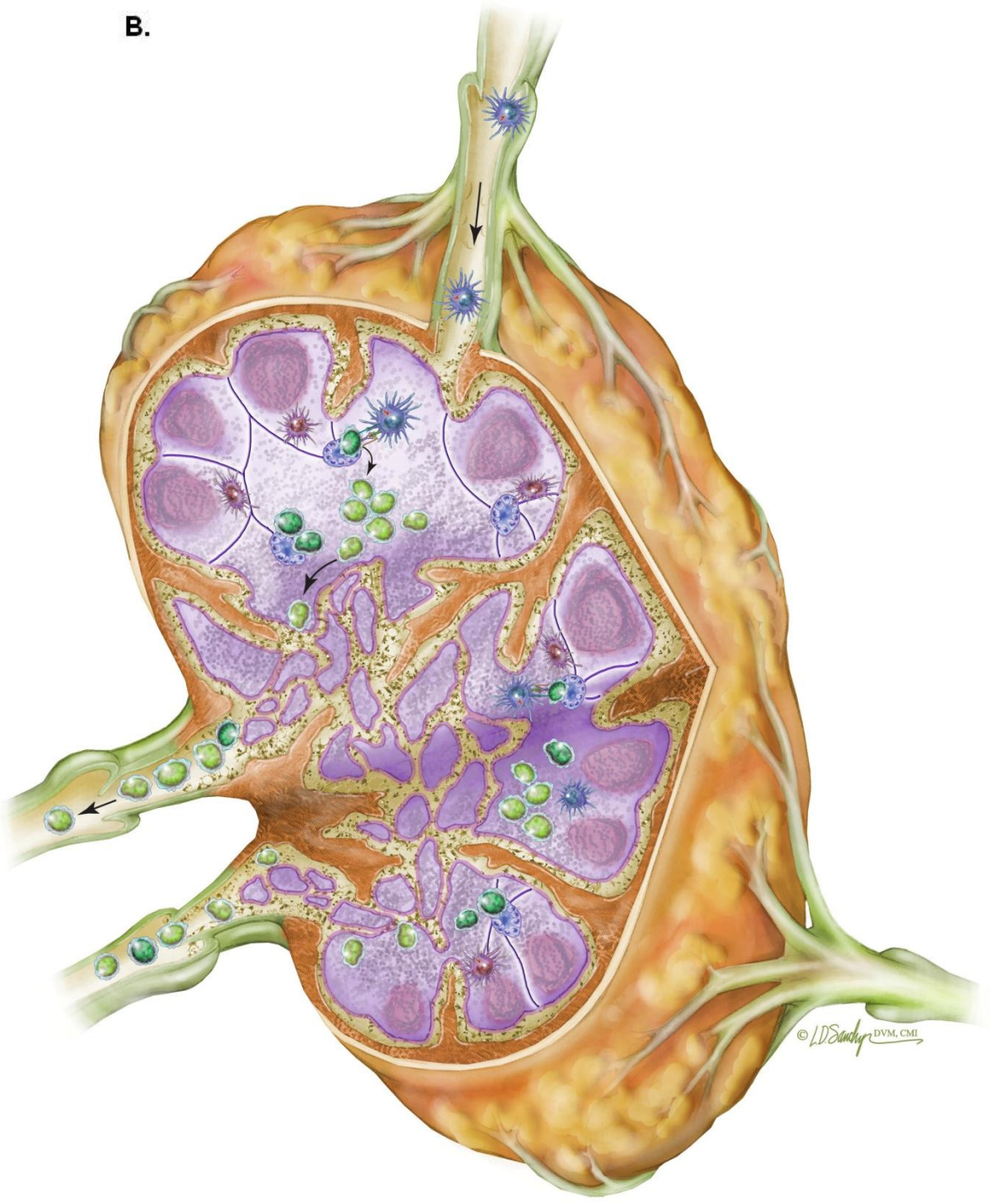
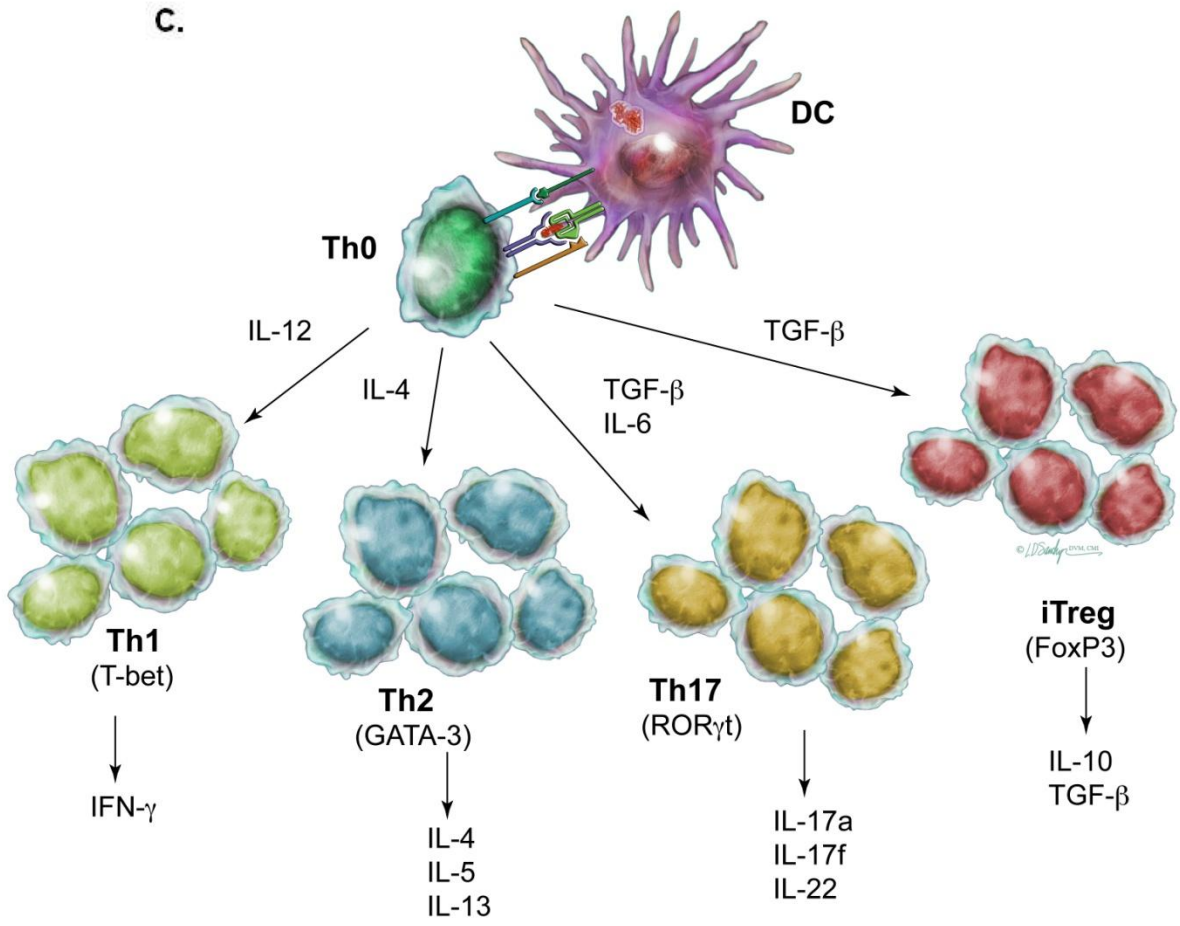


Figure 1.1. The relationship between dendritic cell maturation and regional lymphatic anatomy (continued).



1.4. Antigen Presentation

T lymphocytes respond to antigens derived from foreign proteins, but they are incapable of recognizing these proteins in their native form. Instead, T cells must rely on other cells to process and present peptide fragments derived from the native proteins on their cell surface. The peptides are presented in the context of class I or class II MHC molecules and are recognized by the T cell receptor (TCR) on CD8+ cytotoxic T lymphocytes (CTLs) and CD4+ helper T cells (Th cells), respectively. This process is required for the generation of a T cell response to vaccination, and a brief discussion is warranted to appreciate the essential role DCs play in generating antigen-specific immunity.

MHC class I molecules are expressed by all nucleated cells and are displayed on the cell surface in association with peptides derived from cytosolic proteins. Cytosolic proteins are ubiquitinated to target them for degradation by the proteosome, generating peptide fragments that are translocated to the endoplasmic reticulum (ER) by the transporter associated with antigen presentation (TAP). Once in the ER, the peptides bind to the peptide-binding grooves of MHC class I molecules. Fully assembled peptide-MHC class I complexes exit the ER and are presented on the cell surface (Abbas et al., 2010). This permits primed CTLs to recognize pathogen-infected cells or neoplastic cells and mediate their destruction.

MHC class II molecules are primarily expressed by professional APCs, including DCs, macrophages, and, under certain circumstances, B cells. APCs continually sample their environment via endocytic mechanisms, and the endocytosed proteins are degraded by resident proteases during transport to the MHC class II compartment (MIIC). The resulting peptide fragments bind to the peptide-binding grooves of MHC class II molecules and are transported to

the cell surface (Abbas et al., 2010). Recognition of peptide-MHC class II complexes activates antigen-specific Th cells to help generate the immune response.

One exception to the rule that endogenous antigens are presented on MHC class I and exogenous antigens are presented on MHC class II is a process known as cross-presentation (Kurts et al., 2010). Cross-presented antigens are taken up exogenously but are expressed on MHC class I molecules by APCs. Certain DC subsets are particularly effective at cross-presenting antigen (section 1.7. Dendritic Cell Subsets). Importantly, cross-presentation of antigen by DCs is essential for activating naïve CTLs to respond to vaccination and extracellular pathogens because only DCs express adequate levels of co-stimulatory markers to stimulate these cells. Similarly, DCs are the only APCs capable of efficiently activating naïve Th cells in an MHC class II-dependent manner.

1.5. T Cell Stimulation

Mature DCs in the lymph node are exposed to large numbers of naïve T cells, which continuously circulate from the peripheral blood to the lymph nodes by passing through high endothelial venules (HEVs) in a CD62L-dependent manner. T cells that do not encounter a DC presenting their cognate antigen travel along a sphingosine 1-phosphate (S1P) gradient to exit the lymph node, travel through the efferent lymphatics, and return to the bloodstream via the thoracic duct (Abbas et al., 2010). In contrast, T cells that encounter a DC presenting an MHC molecule bearing a peptide recognized by the TCR (signal 1) are retained in the lymph node. Expression of co-stimulatory molecules (signal 2) and production of cytokines (signal 3) by the mature DC further promote activation and proliferation of the naïve T cell (Joffre et al., 2009).

Effector differentiation of T cells is determined by the paracrine production of DC cytokines (signal 3). As described previously, the cytokines produced by DCs are influenced by the PAMP signature of the offending pathogen. In this way, the DC is able to promote the development of the T cell response that is best equipped to clear the infection (Figure 1.1C). In the example listed above (section 1.2. Dendritic Cell Maturation), TLR synergy increased production of IL-12 by DCs, which enhanced their Th1-polarizing capacity (Napolitani et al., 2005). This type of immunity promotes a strong cell-mediated response consisting of IFN-γ-producing Th1 cells and CTLs, which are important for immunity to intracellular pathogens and tumors (Macatonia et al., 1995). Other PRR-induced, DC-derived cytokines can promote the differentiation of Th2 cells, which secrete IL-4, IL-5, and IL-13 to induce a strong humoral response and potent anti-helminth activity (Mosmann et al., 1986). In addition, DCs activated by different PRRs can also promote the development of Th17 cells, which release IL-17 to protect against extracellular pathogens (Weaver et al., 2007; Olive. 2012).

Immature DCs that have not been exposed to pro-inflammatory molecules also express self-antigens but do not express co-stimulatory molecules (signal 2) or produce cytokines (signal 3). Autoreactive T cells that interact with immature DCs are deleted or enter an anergic state that renders them unresponsive to future antigen encounter (Chappert and Schwartz, 2010). This process occurs continuously under steady state conditions—as evidenced by the anergy induced upon targeting antigen to immature, LN-resident DCs in mice (Hawiger et al., 2001). In this way, immature DCs deplete the pool of autoreactive T cells in the secondary lymphoid tissues. This is a component of a process referred to as peripheral tolerance.

Recently, it has been shown that some DCs can also promote regulatory T cell (Treg) development. These DCs have been exposed to pro-inflammatory cytokines in the context of

inflammation but have not directly contacted a pathogen or its associated PAMPs (Joffre et al., 2009). Such cells, referred to as indirectly activated DCs, increase expression of MHC molecules (signal 1), co-stimulatory molecules (signal 2), and CCR7 but do not increase their cytokine production (signal 3). Therefore, indirectly activated DCs are capable of traveling to the draining lymph node but are only partially activated and, because they do not provide signal 3, are poorly immunogenic (Sporri and Reis e Sousa, 2005). Indirectly activated DCs primarily present self-antigens because they have not directly encountered a pathogen or its derivative components. Providing signal 1 and signal 2 to autoreactive naïve T cells in the absence of signal 3 induces Treg differentiation (Romagnoli et al., 2002). Tregs produce IL-10 and TGF-β to dampen the immune response and prevent immune-mediated disease. Therefore, indirectly activated DCs are referred to as tolerogenic DCs and are actively involved in promoting peripheral tolerance to self-antigen in the face of inflammation (Zanoni and Granucci, 2011). In fact, injection of cytokine-stimulated, tolerogenic DCs bearing self-antigens into mice protects them from developing autoimmunity (Menges et al., 2002).

1.6. Autologous Mixed Leukocyte Reaction

Early experiments demonstrated that mixing the non-T cell fraction of human peripheral blood mononuclear cells with T cells could induce proliferation of T cells *in vitro* in the absence of exogenous antigen (Opelz et al., 1975). The T cell response was termed the autologous mixed lymphocyte reaction (AMLR), and was later shown to require DCs (Nussenzweig and Steinman, 1980). In fact, the ability to initiate the AMLR is a characteristic of DCs, and is dependent upon DC expression of co-stimulatory molecules (Scheinecker et al., 1998). The AMLR is MHC class II-dependent, requires cell-cell contact, and possesses characteristics typical of a normal immune

response, such as memory and specificity (Weksler and Kozak, 1977; Kawamura et al., 1991). The AMLR is thought to represent polyclonal activation of auto-reactive T cells specific for self-antigens presented by DCs (Scheinecker et al., 1998; Amel Kashipaz et al., 2002; Chernysheva et al., 2002; Narendran et al., 2004; Barat et al., 2009). Therefore, this reaction provides a functional assessment of DC stimulatory capacity. The AMLR is deficient in pregnancy, a variety autoimmune disease states, and cancer patients (Indiveri et al., 1983; Gupta and Fanous, 1986; Iwahashi et al., 1996; Angelini et al., 2005).

1.7. Dendritic Cell Subsets

DCs represent a heterogenous population of cells, and DC subsets possess functional specializations (Pulendran et al., 1999). Different DC populations localize to different tissues, are derived from different precursors, express different combinations of cell surface proteins, produce different cytokines upon activation, and possess different capacities for antigen presentation. Although no DC-specific cell surface marker has been identified, these phenotypic differences can be used to define individual DC subsets and correspond to variations in the immune response generated by these cells. Therefore, a detailed understanding of the different DC populations is important to understand their functional capabilities. Recent progress in the increasingly complicated field of DC ontogeny and differentiation, most of which has been performed using murine cells, has been reviewed by several authors (Ju et al., 2010; Liu and Nussenzweig, 2010; Miloud et al., 2010; Kushwah and Hu, 2011). This information has been summarized in Figure 1.2 and Table 1.2.

DC differentiation is initiated in the bone marrow and, although lymphoid progenitors have been shown to generate fully differentiated DCs under appropriate conditions, the majority

of DCs are derived from common myeloid progenitor cells (CMPs). In this case, CMPs differentiate to monocyte and DC progenitor cells (MDPs), which give rise to committed DC progenitor cells (CDPs) and monocytes. In turn, CDPs can differentiate to plasmacytoid DCs or pre-DCs, which leave the bone marrow and give rise to peripheral DC populations.

Broadly, DCs are divided into two subsets depending on ontogeny: conventional and non-conventional DCs. Conventional DCs possess the typical DC form and function and are derived from pre-DCs. They consist of both lymphoid and migratory cells. Lymphoid DCs travel through the blood and enter lymphoid tissues through HEVs in a CD62L-dependent manner, where they reside for prolonged periods of time. Migratory DCs carry antigen from peripheral tissues to lymphoid organs through the afferent lymph in a CCR7-dependent manner. Non-conventional DCs are migratory and consist of plasmacytoid DCs and monocyte-derived DCs. Plasmacytoid DCs are found in lymphoid and non-lymphoid organs and play an important role in innate immunity, as they are capable of producing large quantities of type I interferons in response to viral infections. Monocytes differentiate into a variety of non-conventional DC subtypes in multiple organs under appropriate conditions. This is important to note because many experiments in humans and domestic animals utilize monocyte-derived DCs to model *in vivo*-differentiated DCs. Although these comparisons are not always perfect, monocyte-derived DCs are often preferred due to the relative ease of monocyte purification and high DC yield (Osugi et al., 2002).

Lymphoid DCs are conventional DCs found in the thymus, spleen, and lymph nodes. CD8+CD4+ lymphoid DCs reside in the T cell areas under steady state conditions and specialize in cross-presentation of antigen. Therefore, they efficiently activate CD8+ T cells. CD8- lymphoid DCs localize to the T cell areas only upon stimulation and interact primarily with

CD4+ T cells. CD8- lymphoid DCs are further divided into CD4- cells, which produce IL-12 and IFN-γ, and CD4+ cells, which cannot make IL-12 and appear tolerogenic (Edwards et al., 2003).

Migratory DCs are found in the peripheral tissues in the steady state. Phenotypic differences are present in migratory DC populations from virtually all organs and a complete description of these differences is beyond the scope of this discussion. However, in most cases, CD103+ DCs are conventional (derived from pre-DCs), while CD103- cells represent both conventional and non-conventional DCs. For example, dermal DCs consist of Langerin+CD103+ conventional DCs and Langerin-CD103- non-conventional DCs. CD103+ dermal DCs efficiently cross-present antigen and can promote Th1 and Th17 immune responses, while CD103- dermal DCs appear to be able to initiate a variety of immune responses depending on what stimulus they have encountered. Another non-conventional DC, the Langerhans cell (LC), is present in the epidermis and is self-renewing from primitive macrophages under steady state conditions, or derived from monocytes in the presence of inflammation. LCs travel from the epidermis to the draining lymph node, where they can transfer antigen to CD8+ lymphoid DCs for cross-presentation (Allan et al., 2006).

To make the matter more complicated, different DC subsets have been identified in humans and in mice (Ju et al., 2010; Miloud et al., 2010). This is likely due to a variety of factors, including true species differences, the availability of different tissues and reagents for analysis, and differences in genetic diversity among samples. For example, because human peripheral blood leukocytes are commonly analyzed, many human blood DC subsets have been identified. In contrast, splenic leukocytes are commonly analyzed in mice, and many murine splenic DCs have been characterized. However, recent experiments have begun to resolve some

of these differences and have shown that, although human and murine DC subsets express different combinations of cell surface markers, they may possess conserved transcriptional signatures (Robbins et al., 2008). This suggests that species differences in DC subsets may not be as significant as was previously believed. For example, transcriptome analyses suggest a genetic equivalence of mouse splenic CD11b+CD8- and CD11b-CD8+ DCs with human blood BDCA1+ and BDCA3+ DCs, respectively.

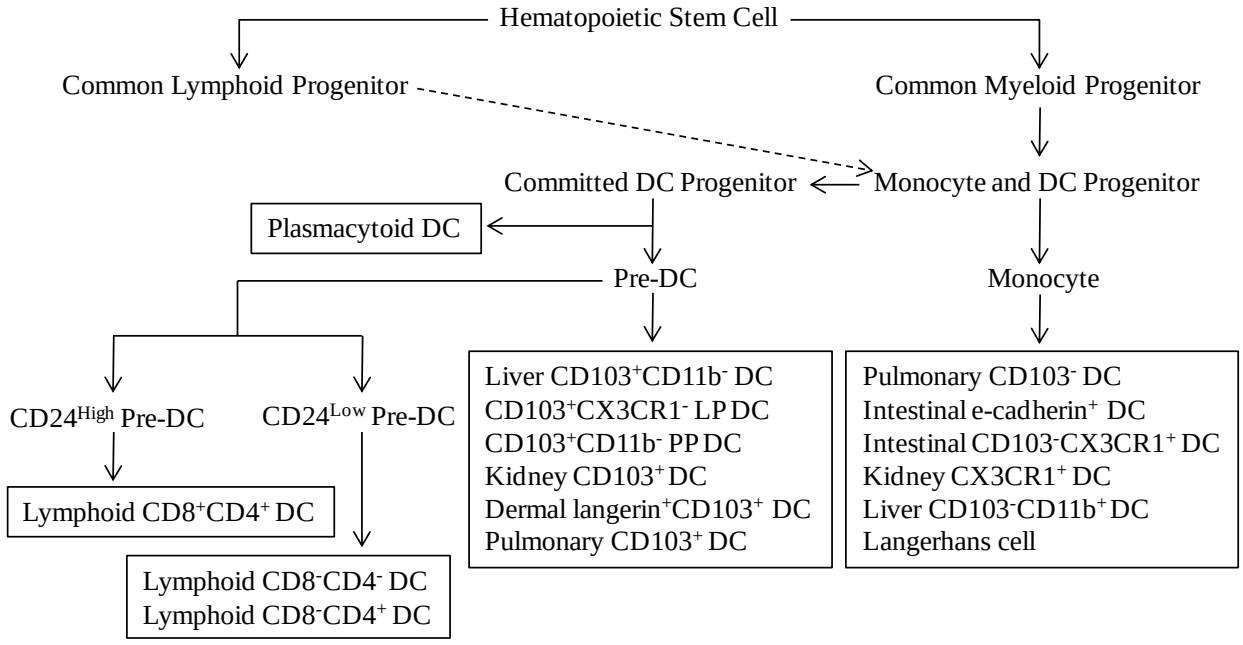


Figure 1.2. Dendritic cell differentiation from hematopoietic stem cells (HSCs).
HSCs give rise to lymphoid and myeloid progenitors. Most DCs are derived from myeloid progenitors, although lymphoid progenitors can differentiate into DCs under the appropriate conditions (dotted line). Myeloid progenitors give rise to monocytes and pre-DCs in the bone marrow. These cells then enter the blood and give rise to DC populations in the lymphoid organs and peripheral tissues. Modified from (Kushwah and Hu, 2011). DC, dendritic cell. LP, lamina propria. PP, Peyer's patch.

Table 1.2. Dendritic cell subtypes organized by tissue.
Modified from (Kushwah and Hu, 2011).
LP, lamina propria. PP, Peyer's Patch.

Lung	**Kidney**
$CD103^{+}$ $CD11c^{hi}$ $CD11b^{-}$ DC	$CX3CR1^{+}$ $CD11b^{+}$ DC
$CD103^{-}$ $CD11c^{hi}$ $CD11b^{+}$ DC	$CX3CR1^{+}$ $CD11b^{-}$ DC
Plasmacytoid DC	$CD103^{+}$ $CX3CR1^{-}$ DC
Intestinal tract	**Lymphoid Tissue**
LP $CD103^{+}$ $CX3CR1^{-}$ $CD11b^{+}$ DC	$CD8^{+}$ $CD4^{+}$ DC
LP $CD103^{-}$ $CX3CR1^{+}$ $CD11b^{+}$ DC	$CD8^{-}$ $CD4^{-}$ DC
PP $CD103^{+}$ $CX3CR1^{+}$ DC	$CD8^{-}$ $CD4^{+}$ DC
E-cadherin^{+} DC	**Skin**
Liver	Langerhans cell
$CD103^{+}$ $CD11b^{-}$ DC	Dermal langerin^{-} $CD103^{-}$ DC
$CD103^{-}$ $CD11b^{+}$ DC	Dermal langerin^{+} $CD103^{+}$ DC
Plasmacytoid DC	

1.8. Dendritic Cell Immunotherapy

As discussed previously (section 1.5. T Cell Stimulation), DCs function as the master regulator of the immune response. As such, the use of these cells to manipulate the immune system therapeutically offers an exciting new treatment approach for a wide range of diseases. For example, traditional vaccines often do not efficiently induce the robust cellular immune response necessary to protect against intracellular pathogens. For this reason, successful vaccination strategies against many devastating human pathogens, including tuberculosis, malaria, and HIV, have not been developed (Plotkin. 2005). Similarly, a strong cell-mediated response is necessary to overcome the immunosuppressive tumor microenvironment and permit CTL destruction of neoplastic cells (Dauer et al., 2008; Chatten and Bathe, 2010). Since DCs have potent cross-presenting and Th1-polarizing capabilities when stimulated appropriately, researchers have speculated that they may be used as adjuvants in vaccines against intracellular pathogens and tumors. In a similar fashion, tolerogenic DCs may be useful to alleviate autoimmune disease, allergy, and transplant rejection.

Traditional vaccines rely on chance encounter of peptide with DCs in the periphery. Novel immunotherapeutic strategies are designed to target specific DC populations, either *in vivo* or *ex vivo* (Palucka et al., 2009). *Ex vivo*-generated DCs permit the generation of large cell numbers under controlled conditions and allow direct delivery of vaccine antigen to these cells for processing and presentation. The antigen-pulsed DCs can be stimulated with PAMPs and cytokines to promote the appropriate immune response before being injected back into the patient to function as a cellular vaccine adjuvant (Inaba et al., 1990; Dhodapkar et al., 1999).

So far, the major focus of DC immunotherapy has been in the area of cancer vaccinology. Immune responses against cancer are often minimal because tumors are derived from host cells,

so the majority of the antigens in a tumor are poorly immunogenic. The primary cell type involved in destroying cancer cells is the CTL. This cell recognizes altered/aberrant self-antigens or oncogenic viral antigens on the MHC class I molecules of tumor cells and mediates their destruction. CTL activation is largely dependent on cross-presentation of tumor antigen by DCs. The importance of Th cells in tumor immunity is less clear, but cytokine production by DC-stimulated Th1 cells likely enhances anti-tumor immunity (Antony et al., 2005; Wieder et al., 2008). DCs also activate NK cells to kill tumor cells that downregulate expression of MHC class I (Fernandez et al., 1999). Therefore, because of their ability to promote a strong anti-cancer immune response in the face of tumor immunosuppressive mechanisms, *ex vivo*-generated DCs provide an attractive cancer vaccine adjuvant alternative (Palucka et al., 2007; McDonnell et al., 2010).

Fortunately, a variety of tumor-associated antigens (TAAs) that can be recognized by the immune system have been identified. These antigens provide the basis for tumor vaccination and include products of mutated genes, abnormally expressed cellular proteins, oncofetal antigens, altered glycolipid and glycoprotein antigens, and antigens of oncogenic viruses (Abbas et al., 2010). DCs can be loaded with TAAs by pulsing them with purified peptides or tumor lysates or by genetic modification before being injected back into the patient to promote an anti-tumor immune response.

Over 100 clinical studies using DC vaccines for cancer have been reported to treat a variety of human cancers, including breast cancer, renal carcinoma, and melanoma, among others (Turnis and Rooney, 2010; Baek et al., 2011; Lesterhuis et al., 2011; Qi et al., 2012). These trials have not used a standard protocol, and the mechanisms for DC isolation and maturation, antigen loading, vaccine dosing, and route of administration have varied greatly.

While these treatments have an impressive safety record, efficacy data has been underwhelming and no optimal protocols have emerged. Evidence of tumor-specific immune responses and moderately prolonged median survival times have been detected in many trials, but complete remissions remain rare.

Sipuleucel-T is an autologous DC-based vaccine that has recently achieved FDA approval for treatment of asymptomatic or minimally symptomatic, metastatic, hormone-refractory prostate cancer. It is prepared by culturing leukocytes from the cancer patient with a recombinant fusion protein consisting of a prostate-specific protein linked to GM-CSF *ex vivo*. The cultured cells, which consist of DCs plus other mononuclear cells, are then infused back into the patient intravenously. A large scale phase III clinical trial with 512 patients indicates that this treatment increased median survival of patients from 21.7 months to 25.8 months (Gupta et al., 2011). Although this response is considered modest, the approval of this product may facilitate the development of other, more effective treatments in the future (Fishman. 2009).

1.9. Equine Dendritic Cells

Dendritic cell research in veterinary species is limited by the use of genetically outbred populations and the dearth of species-specific immunologic reagents. Despite these challenges, considerable progress in the study of equine DCs has been achieved in recent years. Dendritic cells in the horse have been observed *in situ* in a variety of tissues, including normal and inflamed skin, esophagus, cornea, and lymph node (Hamada et al., 1992; Kurotaki et al., 2000; Kurotaki et al., 2002; Espino-Solis et al., 2009; Ledbetter and Scarlett, 2009; Meyer et al., 2010). Peripheral blood DCs have also been described and are enriched in the low density cell (LDC) fraction following Metrizamide™ density centrifugation of transiently adherent peripheral blood

mononuclear cells (PBMCs) (Siedek et al., 1997a). The enriched population consists of mostly large cells with a dendritic morphology that express surface LFA-1 and MHC class I and II, properties that are consistent with DCs from other species. Although expression of CD1b was not detected in LDCs, it was expressed by lymph node DCs, suggesting a mature phenotype for at least a subset of DCs in the equine lymph node (Brigl and Brenner, 2004). Equine lymph node DCs also express the DC-specific integrin CD11c (Espino-Solis et al., 2009). LDCs are more potent stimulators of antigen-specific T cell proliferation and cytotoxicity compared to other APCs (Siedek et al., 1999). LDCs are capable of supporting replication of equine herpesvirus type I (EHV-1), and this infection diminishes their ability to stimulate T cell proliferation to alloantigens (Siedek et al., 1997b).

Isolation of peripheral blood DCs is time-intensive and yields only small numbers of moderately pure DCs. Therefore, a method of generating monocyte-derived DCs by culturing peripheral blood monocytes in medium supplemented with recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) and recombinant equine IL-4 (rEqIL-4) was developed (Hammond et al., 1999). The equine monocyte-derived DCs display many characteristics of human and murine DCs, including a stellate morphology, upregulation of CD86 and MHC class I and II, the capacity to rapidly capture and present soluble and particulate antigens, and the ability to stimulate CD4+ and CD8+ T cells against alloantigens or viral peptides. When compared to other APCs, the monocyte-derived DCs more potently stimulate antigen-specific memory T cell proliferation and cytotoxicity (Soboll et al., 2003a; Rivera and McGuire, 2005).

To facilitate experimentation with equine DCs, improvements in monocyte isolation protocols have been developed to increase purity and yield, including fractionation of PBMCs

over multiple gradients, changes in adherence media and tissue culture dishes, and magnetic selection (Dietze et al., 2008; Steinbach et al., 2009). In addition, some discussion has occurred regarding the optimal culture conditions required to promote DC differentiation from monocyte precursors, including the appropriate cytokines, maturation stimuli, and culture duration. The structure of IL-4 is poorly conserved between humans and horses (62% amino acid identity), and recombinant human IL-4 does not cross-react with equine cells, necessitating the production and use of rEqIL-4 to promote DC differentiation (Vandergrifft et al., 1994; Dohmann et al., 2000). In contrast, GM-CSF is more highly conserved between these species (76.4% amino acid identity), and rHuGM-CSF can stimulate equine monocytes (Hammond et al., 1999). However, the equine GM-CSF gene contains a 24-nucleotide extended open reading frame that has not been described in any other species examined to date. The nucleotide addition does not fall within the predicted active regions of the cytokine, and recombinant equine GM-CSF (rEqGM-CSF) is still capable of stimulating robust proliferation of human cells (Kaushansky et al., 1989; Mauel et al., 2006). Nevertheless, rEqGM-CSF does stimulate the growth of more colonies of equine bone marrow cells in agar compared to rHuGM-CSF (Vecchione et al., 2002). It is important to note that some researchers have used rHuGM-CSF while others have used rEqGM-CSF to produce equine monocyte-derived DCs, and that these cytokines have been used at differing concentrations. Unfortunately, no systematic comparisons between the ability of these two cytokines to promote the differentiation of monocytes into DCs have been performed.

Additional experiments revealed that, compared to monocytes, DCs downregulate CD14 and upregulate the macrophage mannose receptor (CD206), MHC class II, CD1a, and CD1b. Initial attempts to stimulate monocyte-derived DCs with the TLR ligands LPS and poly(I:C) did not consistently produce changes in cell surface marker expression normally associated with a

mature DC phenotype (Mauel et al., 2006). However, although IL-4 itself may induce some degree of DC maturation, stimulation with LPS increased expression of IL-12 mRNA (Dietze et al., 2008). Many questions exist regarding the best way to define the maturation state of equine DCs, and this remains an active area of research.

Complete differentiation of equine monocyte-derived DCs has been reported to occur after 6-7 days of culture with rEqIL-4 and rEqGM-CSF (Mauel et al., 2006). This conclusion was based upon morphologic and cell surface marker expression, although no supporting data was shown. When the DCs were cultured in rEqIL-4 and rHuGM-CSF, optimal expression of the DC-related markers EqWC2, MHC class II, and CD86 were detected at days 3 and 4 of culture (Dietze et al., 2008). The DC markers were down-regulated after 4 days as reported for human monocyte-derived DCs, possibly due to culture overgrowth (Steinbach et al., 1998). The reason for this discrepancy in optimal culture duration may be due to differences in the isolation protocol, the source of GM-CSF, or variations in other culture conditions.

Monocyte-derived DCs are a valuable research tool for studying the equine immune response. In particular, equine DCs have been used to define differences between the immune systems of foals and adult horses and to advance our understanding of the equine immune response to several viral pathogens. For example, DCs were used to identify an EHV-1 protein containing an MHC class I-restricted epitope, a finding that may promote the development of an efficacious EHV-1 vaccine (Soboll et al., 2003b). Also, the discovery that equine influenza virus infection inhibits the differentiation of equine DCs may offer insight into the pathogenesis of the virus *in vivo* (Boliar and Chambers, 2010).

Foal monocyte-derived DCs are less mature than their adult counterparts, as evidenced by a decrease in the number of CD14-CD1b+CD86+ mature DCs and lower expression of MHC

class II (Flaminio et al., 2009; Merant et al., 2009). Foal DCs also produce lower levels of a variety of cytokines at steady state, including TNF-α, IL-10, MCP-1, and TGF-β. Upon stimulation with cytosine-phosphate-guanosine oligodeoxynucleotide (CpG-ODN), a TLR 9 ligand, foal DCs are unable to upregulate expression of IL-12p40 and IFN-α mRNA. Compared to adult DCs, foal DCs differentially express multiple genes in response to infection with *Rhodococcus equi* (*R. equi*) (Heller et al., 2010). Infected foal DCs express higher levels of the IRF-1 transcription factor and IL-12 compared to adult cells (Flaminio et al., 2009). These findings have contributed to our understanding of rhodococcal infections in foals and may lead to novel prophylactic or therapeutic interventions.

Because monocyte-derived DCs are easily generated in large numbers and possess potent immunostimulatory properties, they may be used therapeutically to modulate the equine immune response (Vecchione et al., 2002). For example, equine DCs have generated interest as a vaccine against equine sarcoid and squamous cell carcinoma. Equine DCs do not produce adverse reactions when loaded with tumor antigen and administered intra-dermally as a DC vaccine, but such vaccines have not yet been evaluated for efficacy (Steinbach et al., 2009).

1.10. Study Aims

Although this comprehensive review highlights the considerable contributions to our understanding of equine DCs over the past two decades, the study of these cells is in its infancy when compared to those of humans. However, the availability of reagents to study the equine immune response has increased greatly in recent years. Furthermore, veterinary medicine is an increasingly sophisticated field; we have begun to understand many disease processes in great detail, and many of our diagnostic and treatment modalities are on par with those used by

physicians. Therefore, it is not unreasonable to expect that DC immunotherapy may represent a valid treatment option for a variety of disease processes in the horse. DC-adjuvanted vaccines may be used to prevent infectious diseases such as equine herpesvirus 1 (EHV-1) and *R. equi* or to treat non-infectious diseases including neoplasia and immune-mediated conditions. This dissertation was motivated by the desire to further develop our understanding of equine DCs. In particular, I have endeavored to characterize the phenotype and function of equine monocyte-derived DCs. In doing so, I hope to validate the use of DCs as a tool to study the equine immune system and, possibly, as a novel immunotherapeutic agent.

1.11. References

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CHAPTER 2:

EX VIVO GENERATION OF MATURE EQUINE MONOCYTE-DERIVED DENDRITIC CELLS*

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2.1. Summary

Dendritic cells (DCs) are innate immune cells specialized in antigen detection and presentation. They perform an essential role in initiating and guiding the immune response, the direction of which largely depends upon DC activation state. The objective of this study was to generate mature equine monocyte-derived DCs and, in doing so, to develop a method for measuring the activation state of these cells. Equine DCs were stimulated with UV-inactivated *Escherichia coli* (*E. coli*), and the activation status was measured by analyzing cell surface marker expression, cytokine production, and endocytic capacity. Comparisons for each parameter measured were performed between macrophages, non-stimulated DCs, and stimulated DCs. Equine monocyte-derived DCs may be distinguished from macrophages based on cell surface expression of MHC class II ($p < 0.0001$) and CD206 ($p < 0.0001$), their capacity for endocytosis of FITC-dextran ($p < 0.05$), and production of TNF-α upon stimulation ($p < 0.001$). Furthermore, stimulated DCs can be distinguished from non-stimulated DCs based on increased cell surface expression of MHC class II ($p < 0.0001$) and upregulation of pro-inflammatory cytokine mRNA, particularly IL-12/IL-23p40 ($p < 0.05$) and IL-23p19 ($p < 0.05$). The ability to measure DC activation state will facilitate future investigations of equine DC function.

2.2. Introduction

Dendritic cells (DCs) represent a heterogeneous cell population specialized in immune surveillance, antigen presentation, and initiation of the adaptive immune response. The subtype, immunologic microenvironment, and activation state of a DC determines the nature of the adaptive immune response produced, including a variety of effector responses, anergy, or tolerance (Steinman and Hemmi, 2006). The pivotal role of DCs in directing the immune response has generated interest in DC biology and its relevance to many aspects of immunology. DCs possess potential for use as immunotherapeutic agents with respect to vaccination, transplantation, cancer, autoimmunity, and allergy (Steinman and Banchereau, 2007).

Dendritic cells are present in most tissues and comprise a small fraction of the leukocytes throughout the body, representing only about 1% of peripheral blood mononuclear cells (Sato and Fujita, 2007). Consequently, DCs are isolated in small numbers from biological samples, making them difficult to analyze (Peña-Cruz et al., 2001). DC studies are further complicated by the presence of multiple subtypes, including conventional DCs as well as type I interferon-producing plasmacytoid DCs (Shortman and Naik, 2007). Characterization of DCs has been greatly facilitated by *ex vivo* methods of cell generation, such as the incubation of peripheral blood monocytes with IL-4 and GM-CSF (Sallusto and Lanzavecchia, 1994). Historically, such monocyte-derived DCs have served to model the conventional, migratory DC. Coventional DCs reside in the peripheral tissues in an immature state and serve as the sentinels of the immune system, taking up antigen and scanning the body for evidence of infection. They express cell surface receptors that recognize invading pathogens, pro-inflammatory host molecules, and damaged host cells (Adams et al., 2005). Once activated by these so-called “danger signals”, migratory DCs adopt a more mature phenotype and home to the draining lymph node by

increasing expression of the chemokine receptor CCR7 (Förster et al., 2008). DC maturation is characterized by a shift from antigen detection to antigen presentation, a process that involves upregulation of MHC class II, co-stimulatory molecules and pro-inflammatory cytokines, and a decrease in both endocytic capacity and expression of endocytic receptors such as CD206 (Banchereau and Steinman, 1998; Jeras et al., 2005; Steinman and Hemmi, 2006; Macagno et al., 2007).

The generation of DCs from equine monocytes has been described previously (Hammond et al., 1999; Mauel et al., 2006; Dietze et al., 2008). Research in this area has been complicated by difficulties in measuring DC activation status. *Ex vivo* generated equine DCs represent a mixed population of immature and mature (activated) cells (Hammond et al., 1999). It has been postulated that equine monocyte-derived DCs are refractory to stimulation by individual toll-like receptor (TLR) ligands under experimental conditions (Mauel et al., 2006). Our lab has demonstrated that equine monocyte-derived DCs from adult horses respond to stimulation by cytosine-phosphate-guanosine oligodeoxynucleotide (CPG-ODN), a TLR 9 ligand (Flaminio et al., 2007). More recently, equine DCs have been shown to increase expression of IL-12 upon incubation with LPS (Dietze et al., 2008).

Our objective in the present study was to further characterize the maturation of equine monocyte-derived DCs. Human DCs have demonstrated increased activation when stimulated with multiple TLR ligands, suggesting a synergistic effect when signaling through multiple pattern recognition receptors (PRRs) (Napolitani et al., 2005). Therefore, we hypothesized that stimulating equine DCs with UV-inactivated *Escherichia coli* (*E. coli*) would induce a more mature DC phenotype by exposing them to multiple pathogen-associated molecular patterns (PAMPs) present on the surface of the intact bacteria, rather than attempting to stimulate the

cells with single, purified TLR ligands. We predicted that activation of the DCs would be evidenced by alterations in cell surface expression, cytokine production, and endocytic activity. We anticipate this work will facilitate future studies that lead to a greater understanding of the equine immune system and to the use of equine DCs in immunotherapeutic techniques.

2.3. Materials and Methods

2.3.1. Monocyte-derived macrophage and dendritic cell culture

Equine monocyte-derived DCs were prepared according to a protocol similar to that described previously (Hammond et al., 1999; Flaminio et al., 2007). Briefly, peripheral blood was collected into 10 mL heparinized vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by jugular venipuncture from healthy adult horses according to an approved Institutional Animal Care and Use Committee (IACUC) protocol. Peripheral blood mononuclear cells (PBMCs) were isolated using 1077 Ficoll-paque (Amershan Biosciences, Piscataway, NJ) density centrifugation. PBMCs were plated into 24-well flat-bottom tissue culture plates (Becton Dickinson, Franklin Lakes, NJ) at a concentration of 1×10^7 PBMCs per well in 500 µL of DMEM-F12 complete medium (Gibco-Invitrogen Corporation, Grand Island, NY) with 10% bovine growth serum (Hyclone, Logan UT), antibiotics and antimycotics (Gibco-Invitrogen Corporation, Grand Island, NY). The cells were allowed to adhere for 2 h at 5% CO_2 and 37ºC. Non-adherent cells were removed by gently washing the well two times with DMEM-F12 media. DCs were produced (Figure 2.1) by culturing the adherent monocyte-enriched population (approximately 3×10^6 to 5×10^6 cells) in complete medium supplemented with 10 ng/mL recombinant equine IL-4 (rEqIL-4) (kindly provided by Dr. David Horohov, University of Kentucky, Lexington, KY) and 50 ng/mL recombinant human GM-CSF (rHuGM-CSF) (R&D Systems, Minneapolis, MN). For

comparison, macrophages were generated by culturing monocytes in complete medium without exogenous cytokines.

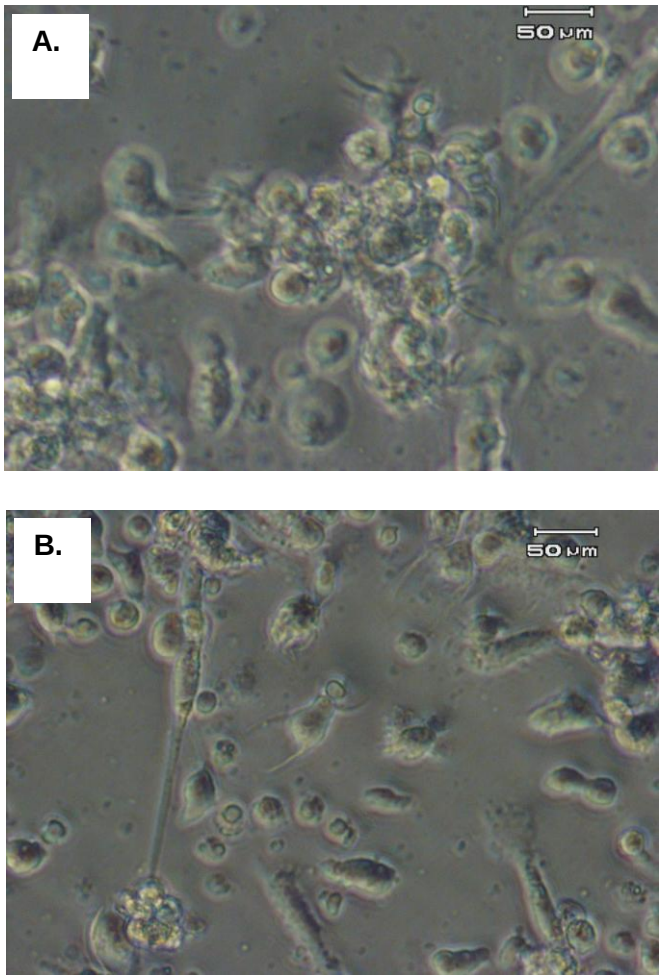


Figure 2.1. Photomicrograph of equine monocyte-derived dendritic cells.
Equine monocyte-derived dendritic cells were grown in tissue culture containing complete medium supplemented with rEqIL-4 and rHuGM-CSF. Photomicrographs of DCs were taken on day 5 of culture. Note the loosely adherent, veiled cells with multiple cellular projections (A) and the heterogeneous nature of the cell population (B).

2.3.2. Inactivation of Escherichia coli

A blood agar plate streaked with *Escherichia coli* (*E. coli*) isolated from normal horse manure was obtained from the Animal Health Diagnostic Center (AHDC) at the Cornell University College of Veterinary Medicine. This sample was confirmed to be 100% pure *E. coli* by biochemical analysis at the AHDC. The *E. coli* was inoculated into 300 mLs of trypticase soy broth (TSB) and grown overnight at 37ºC under agitation. A bacterial count was obtained by growing serially-diluted pour plates. The bacterial culture was run through a CiderSure purifier (Oesco, Inc. Conway, MA) in the laboratory of Dr. Randy W. Worobo (Department of Food Science and Technology, Cornell University). The CiderSure is a powerful ultraviolet (UV) light source used commercially as a substitute for pasteurization of apple cider. This technique was used to inactivate the bacteria while preventing denaturation or alteration of the bacterial structure. Cultures of the inactivated *E. coli* failed to produce any colonies. Aliquots of the inactivated bacteria were made in 10% glycerol and stored at -80ºC.

2.3.3. Cell stimulation

Monocyte-derived macrophages or DCs were stimulated on day 3 of culture by adding 3 x 10^7 inactivated *E. coli* per well (approximately 10 bacteria per cell) in a 24-well tissue culture plate. Cells were harvested at the appropriate time point for each particular experiment. Day 3 of culture was chosen for stimulation based on preliminary studies comparing cell surface marker expression between cells stimulated on days 3, 4, or 5 (data not shown) and a kinetic analysis of MHC class II expression in non-stimulated MOs and DCs (Figure 2.2).

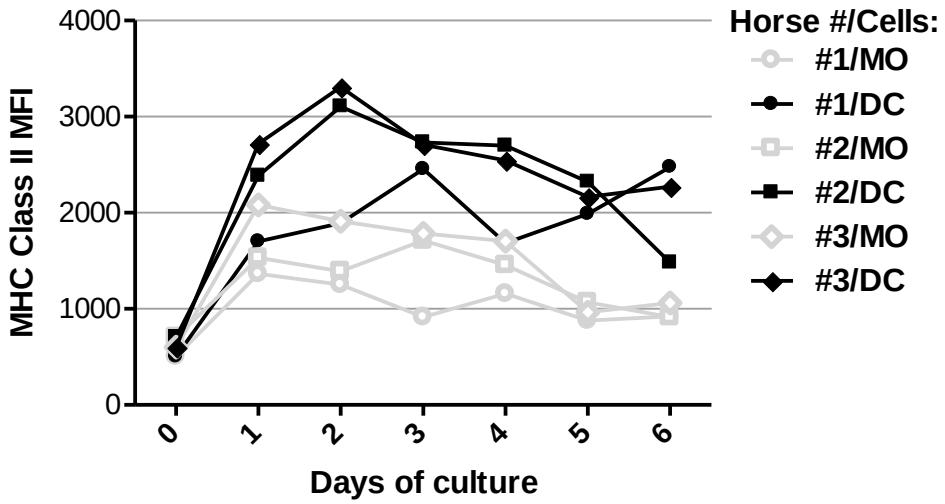


Figure 2.2. Kinetics of MHC class II expression by equine DCs and MOs. DCs or MOs from 3 different horses were cultured for 0 to 6 days and analyzed daily for surface expression of MHC class II by flow cytometry. MO, macrophage. DC, dendritic cell. MFI, mean fluorescence intensity.

2.3.4. Flow cytometry

For each of 3 horses, 10 macrophage wells and 10 DC wells were plated in 24-well tissue culture plates, as described. Half of the wells (5 macrophage wells and 5 DC wells) were stimulated with UV-inactivated *E. coli* on day 3, producing 4 treatment groups: non-stimulated macrophages (MO NS), stimulated macrophages (MO stim), non-stimulated DCs (DC NS), and stimulated DCs (DC stim) (Figure 2.3). All wells were harvested on day 5 with 500 µL of Versene (Gibco-Invitrogen, Carlsbad, CA) and spun down in a microcentrifuge at 167 x g for 10 min. The supernatant was collected and stored at -80ºC for cytokine analysis. Approximately 1 x 10^6 cells were incubated in 10% normal goat serum for Fc receptor blocking, washed with phosphate buffered saline (PBS), labeled with fluorophore-conjugated antibody, washed again, and analyzed with a FACSCalibur® flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Antibodies used included ALEXA 488-conjugated mouse anti-horse MHC class II (hybridoma clone CZ11, kindly provided by Dr. Douglas Antczak, Cornell University, Ithaca, NY) and mouse anti-human CD206-PE (hybridoma clone 3.29B1.10, Beckman Coulter, Fullerton, CA) (Lunn et al., 1998; Steinbach et al., 2005). The cells were displayed on a dot plot, and the appropriate leukocyte subpopulation was gated according to size and granularity. The gated area has been confirmed to include CD172a-positive cells in our previous studies (Flaminio et al., 2007).

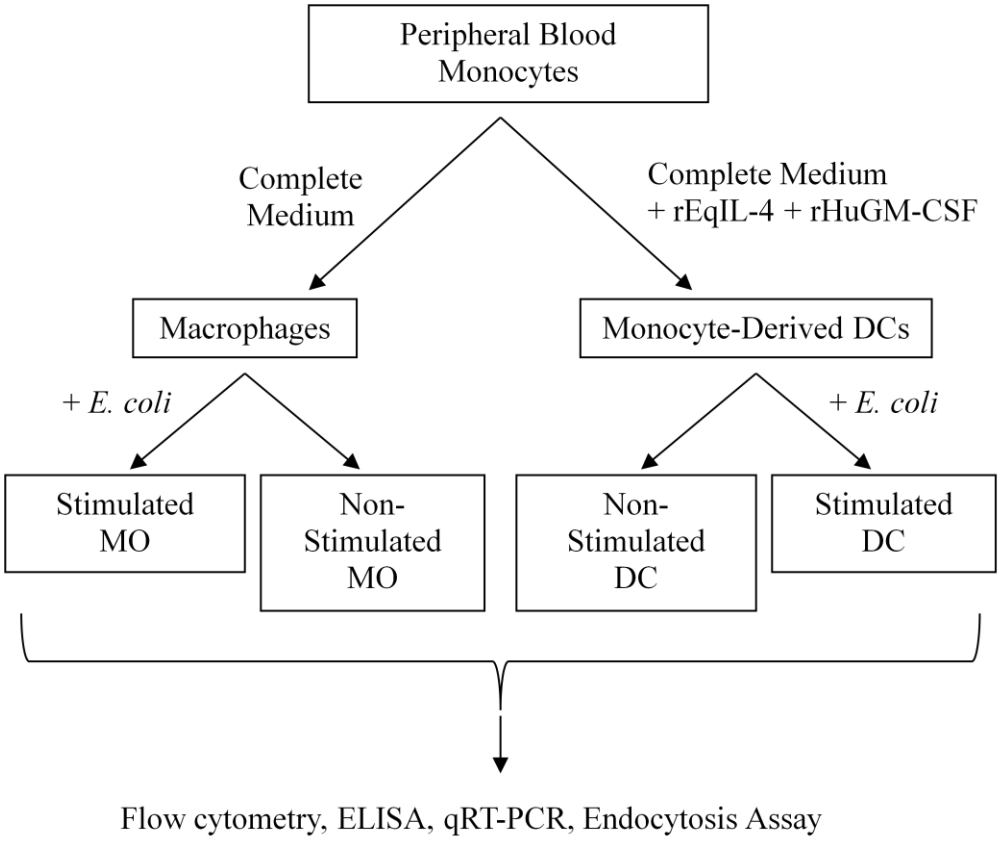


Figure 2.3. Protocol for stimulating macrophages and dendritic cells.
Equine monocytes were purified from peripheral blood by 1077 Ficoll-paque density centrifugation and plastic adherence. DCs were produced by culturing monocytes in complete medium supplemented with rEqIL-4 and rHuGM-CSF. Macrophages were produced by culturing monocytes in complete medium with no added cytokines. After 3 days of culture in 24-well tissue culture plates, half of the DCs and MOs were stimulated with *E. coli* to produce 4 treatment groups: MO NS, MO Stim, DC NS, DC Stim. Cells and supernatants were harvested at 12h and 18h and analyzed for surface marker expression, cytokine production, and endocytic activity. MO, macrophage. DC, dendritic cell. NS, Non-stimulated. Stim, Stimulated.

2.3.5. Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Quantitative RT-PCR was used to measure expression of cytokines and cell surface molecules. For each of 2 horses, 8 macrophage wells and 8 DC wells were plated. Half of the wells (4 macrophage wells and 4 DC wells) were stimulated on day 3. For each of the 4 treatment groups, 2 wells were harvested at 12 and 18 hours of stimulation, which were determined to be the period interval for peak expression based on kinetics experiments (data not shown). Total RNA was isolated using the RNeasy® kit (Qiagen, Valencia, CA), and RNA yield and quality was measured by spectrophotometry. The RNA was treated with DNase (Gibco-Invitrogen, Carlsbad, CA) and frozen at -80ºC until use. For each sample, 10 ng of RNA was used to measure expression of cytokines (IL-12p35, IL-23p19, IL-12/IL-23p40, IL-10) and cell surface markers (CCR7, CD40, CD86). Primers and probes for IL-12p35, IL-12p40, IL-10, and β-actin have been published previously (Flaminio et al., 2007). Primers and probes for IL-23p19, CCR7, CD40, and CD86 were constructed based on GenBank equine genomic sequences (Table 2.1). Samples were measured in triplicate using Taqman® one-step RT-PCR master mix reagents, specific primers and probes, and the ABI Prism® 7700 Sequence Detection System (AB Biosystems, Foster City, CA). Data analysis was performed by normalizing the values based on an endogenous control (β-actin expression), and calculating the fold-change of stimulated samples over non-stimulated samples ($2^{-\Delta\Delta CT}$). The efficiency of amplification of the target genes and the reference gene were similar, with linear curve slopes < 0.01.

Table 2.1. Primer and probe sequences used to measure cytokine and cell surface marker expression by qRT-PCR.

Gene	Primer and Probe Sequence	GenBank Accession #
IL-23 p19	5'-AGCAACCCTGACCCCTTAAAG-3'	NM_001082522
	5'-CATCTTAGCATTGCTGAGCCAT-3'	
	5'-6FAM-AGCAGCTTTAAGGATGGCACCCACATC-TAMRA-3'	
CCR7	5'-CCTGTGCCAAGATGAGGTCA-3'	XM_001500181
	5'-AGTTCCGGACATCCTTCTTGAA-3'	
	5'-6FAM-TTACATCGGCGACAACACCACGGT-TAMRA-3'	
CD40	5'-AACAGGACGTCTCATCGTGGTT-3'	AY514017
	5'-TTTATTTAGCCAGTCCCCTGTTG-3'	
	5'-6FAM-TGAACAGCTGGAACTCACTGAACTGTCCA-TAMRA-3'	
CD86	5'-TGAGCCAACGGAGACACTGAT-3'	CD468511
	5'-CTGTAATCCAGCGGATGTGGT-3'	
	5'-6FAM-CACGTGAGAAAGCCCAGCATGAACAA-TAMRA-3'	

2.3.6. ELISA

Supernatants collected from the cells harvested for flow cytometric analysis were stored at -80ºC. TNF-α expression was measured in these samples using the DuoSet ELISA Development kit (R&D Systems, Minneapolis, MN). A concentration standard curve was generated from reference dilutions and their respective optical density (O.D.) values. The concentrations of TNF-α in each testing sample were determined from the standard curve according to individual O.D. The wells were read using a spectrophotometer at 450nm, and data analyzed using 'Ascent Software' (Thermo Electron Corporation, Vantaa, Finland).

2.3.7. FITC-dextran endocytosis assay

Cells from peripheral blood of 2 horses were used to grow 3 macrophage wells and 6 DC wells as described. Half of the DC wells were stimulated with UV-inactivated *E. coli* on day 3 of culture. All cells were harvested on day 4 of culture by gentle scraping with a rubber policeman in 500 µL of cold PBS. The ability of these cells to uptake FITC-labeled dextran was assayed in a manner similar to that published previously (Sprague et al., 2005). Briefly, all cells were washed once and resuspended in DMEM F12 medium with 1% Bovine Growth Serum (BGS). The cells were split into Eppendorf microcentrifuge tubes (Eppendorf, Hamburg, Germany), with 1 x 10^6 cells per tube. All tubes were incubated on ice for 30 min before adding FITC-dextran (MW 70,000) (Invitrogen-Molecular Probes, Carlsbad, CA) to a final concentration of 500 µg/mL. Cells were incubated on ice (control) or at 37ºC on a spinning rack for 30 min, washed 2 times in 1 mL of ice-cold DMEM F12 medium with 1% BGS, and resuspended in PBS for flow cytometric analysis.

2.3.8. Statistical analysis

These experiments were designed to compare the differentiation of equine monocytes into DCs or macrophages, and to analyze the response of DCs to stimulation with UV-inactivated *E. coli*. To minimize irrelevant variability, multiple samples were taken from a small number of horses (3 for flow cytometry and ELISA, 2 for qRT-PCR and endocytosis assays). As suspected, the horse from which the cells were drawn made a significant difference in some of the results. This was accounted for in the 3-way ANOVA model used to compare samples. For each treatment group and each outcome, a Shapiro-Wilk test was used to confirm that most of the data were Gaussian, and then all data were treated as Gaussian. A Tukey's HSD test was used to compare the combinations implied by the interaction term among the treatments. A p-value of 0.05 (2-sided) was used for significance for all tests. All statistical analyses were performed using Statistix® 8.0 software (Analytical Software, Tallahassee, FL).

2.4. Results

2.4.1. Effect of E. coli stimulation on antigen presentation, costimulation, and chemokine receptor expression

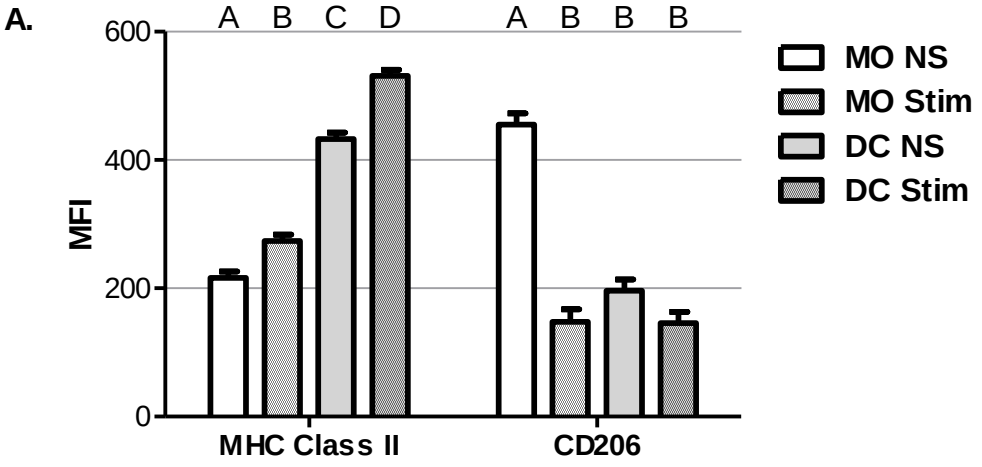
Flow cytometric analysis revealed that DCs expressed more MHC class II than macrophages ($p < 0.0001$), and stimulation of macrophages or DCs increased expression of MHC class II ($p < 0.0001$) (Figure 2.4A). Pairwise comparisons revealed that each of the treatment groups expressed MHC class II at a statistically distinct level ($p < 0.05$ for each). Furthermore, the increase in MHC class II expression upon stimulation was greater in DCs than in macrophages ($p < 0.05$). Expression of CD206 was higher in non-stimulated macrophages

than all other treatment groups ($p < 0.0001$), and this expression level dropped sharply upon stimulation.

Quantitative RT-PCR analysis revealed that CCR7 mRNA levels in DCs did not respond to stimulation (Figure 2.4B). Curiously, CCR7 mRNA levels increased slightly in stimulated macrophage samples at both 12 and 18 hours of stimulation ($p < 0.05$). This increase was quite modest, however, and was not significantly different from the levels of mRNA detected in stimulated DC samples ($p > 0.05$). qRT-PCR analysis did demonstrate an approximately 2-fold increase in expression of CD86 in macrophages stimulated for 18 hours and DCs stimulated for both 12 and 18 hours ($p < 0.05$) (Fig 2.4C). A 2.5-fold increase in CD40 mRNA levels was measured in DCs following 18 hours of stimulation ($p < 0.05$), but a comparable increase was not detected upon stimulation of macrophages (Figure 2.4D).

Figure 2.4. Effect of *E. coli* stimulation on equine macrophage and DC surface marker expression. Stimulated cells were incubated with *E. coli* for 48 h before flow cytometric analysis and for 12 or 18 h before qRT-PCR analysis. Data represents least-squares means and pooled standard errors. (A) Expression of MHC class II and CD206 measured by flow cytometry. n = 15 (14 for MO Stim). (B) Expression of CCR7 mRNA measured by qRT-PCR. n = 4. (C) Expression of CD86 mRNA measured by qRT- PCR. n = 4. (D) Expression of CD40 mRNA measured by qRT-PCR. n = 4. MO NS, non-stimulated macrophages. MO Stim, stimulated macrophages. DC NS, non-stimulated dendritic cells. DC Stim, stimulated dendritic cells. MFI, mean fluorescence intensity.

Figure 2.4. Effect of *E. coli* stimulation on equine macrophage and DC surface marker expression (continued).



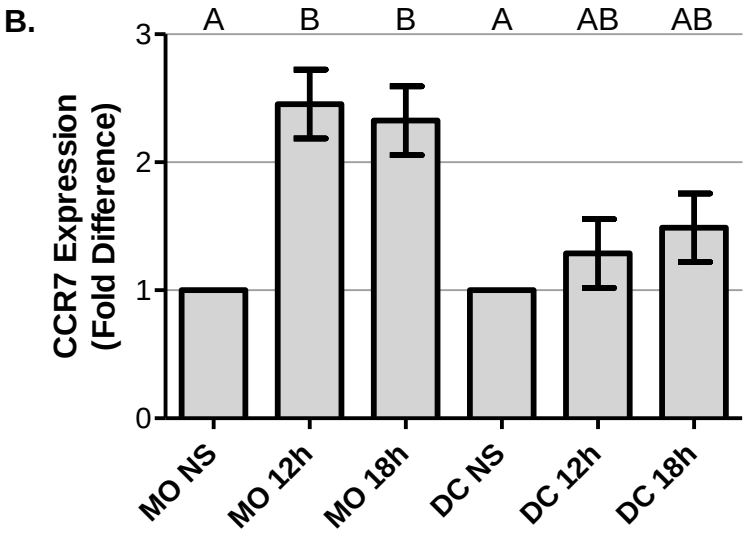
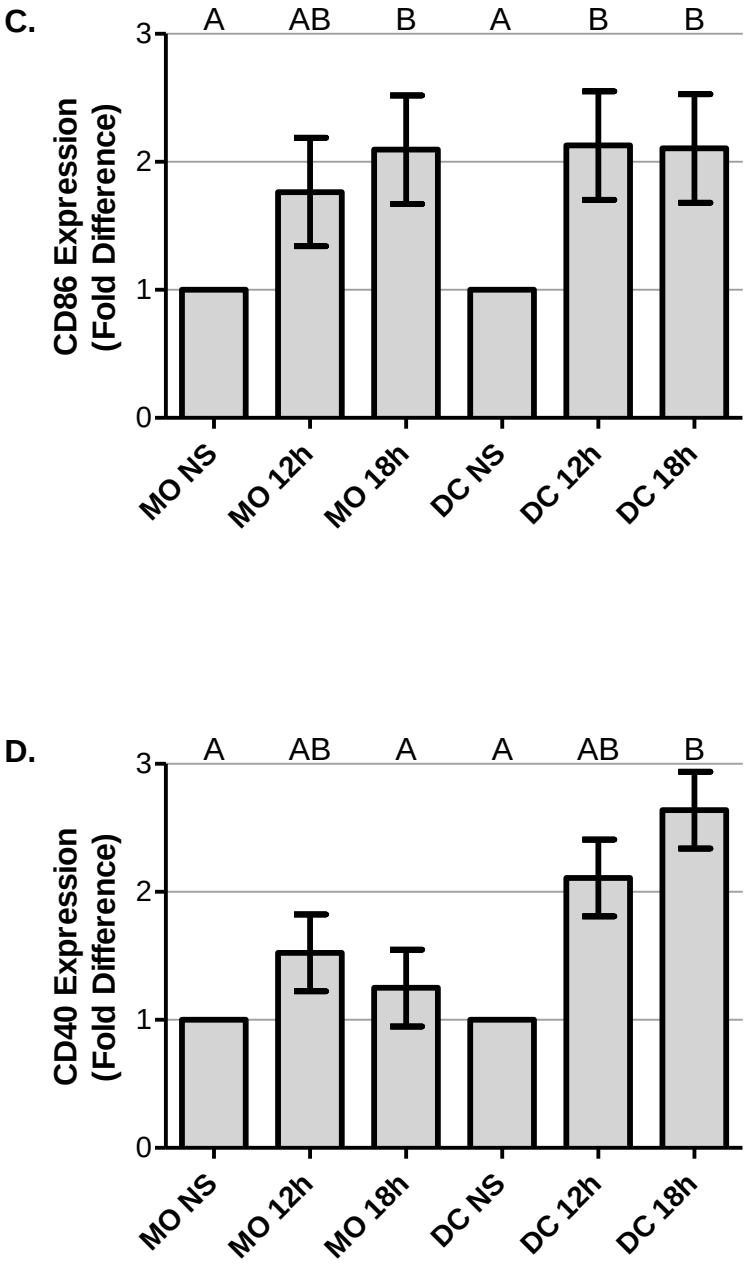


Figure 2.4. Effect of *E. coli* stimulation on equine macrophage and DC surface marker expression (continued).



2.4.2. Effect of stimulation on cytokine expression

Non-stimulated macrophages and DCs produced low levels of TNF-α (Figure 2.5A). Upon stimulation with *E. coli*, macrophages exhibited a 24-fold increase ($p < 0.001$) in TNF-α production. While a 19-fold increase in production of TNF-α by stimulated DCs in comparison to non-stimulated DCs was also noted, this difference was statistically insignificant ($p > 0.05$) and was lower than the upregulation of TNF-α seen in macrophages ($p < 0.05$).

Other cytokines were measured by qRT-PCR analysis of mRNA transcripts. The p40 subunit of IL-12 and IL-23 (Figure 2.5B) was upregulated upon stimulation of macrophages and DCs ($p < 0.05$). The degree of upregulation (48- to 118-fold) was similar in both cell types, and did not vary between the two time points measured. DCs and macrophages slightly increased expression of the IL-12p35 transcript upon stimulation (Figure 2.5C). The upregulation was greatest (2.7-fold) in DCs stimulated for 12 hours ($p < 0.05$). The p19 subunit of IL-23 was upregulated significantly (9- to 18-fold) in macrophages and DCs stimulated for 12 and 18 hours ($p < 0.05$) (Figure 2.5D). Modest increases in IL-10 production (Figure 2.5E) were seen in stimulated DCs ($p < 0.05$) at both time points measured. While a similar increase in IL-10 transcription was not measured in stimulated macrophages, the expression levels in stimulated macrophages were equivalent to those of stimulated DCs.

Figure 2.5. Effect of *E. coli* stimulation on equine macrophage and DC cytokine expression. Data represents least-squares means and pooled standard errors. (A) TNF-α production was measured by ELISA in supernatant collected from stimulated or non-stimulated macrophages and DCs after 48 h of stimulation. n = 12. Changes in expression of (B) IL-12/IL-23p40, (C) IL-12p35, (D) IL-23p19, and (E) IL-10 mRNA transcripts in macrophages and DCs were measured at 0, 12 and 18 h of stimulation. n = 4 for qRT-PCR data. MO NS, non-stimulated macrophages. MO Stim, stimulated macrophages. DC NS, non-stimulated dendritic cells. DC Stim, stimulated dendritic cells.

Figure 2.5. Effect of *E. coli* stimulation on equine macrophage and DC cytokine expression (continued).

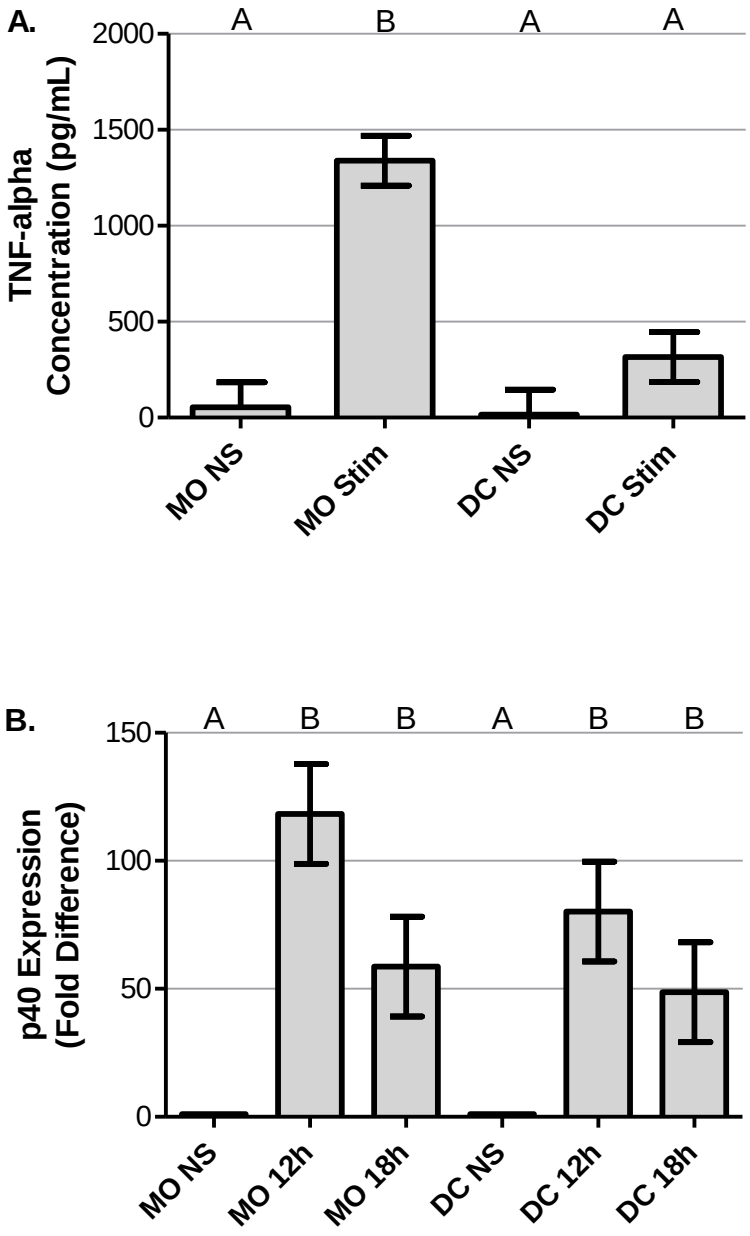


Figure 2.5. Effect of *E. coli* stimulation on equine macrophage and DC cytokine expression (continued).

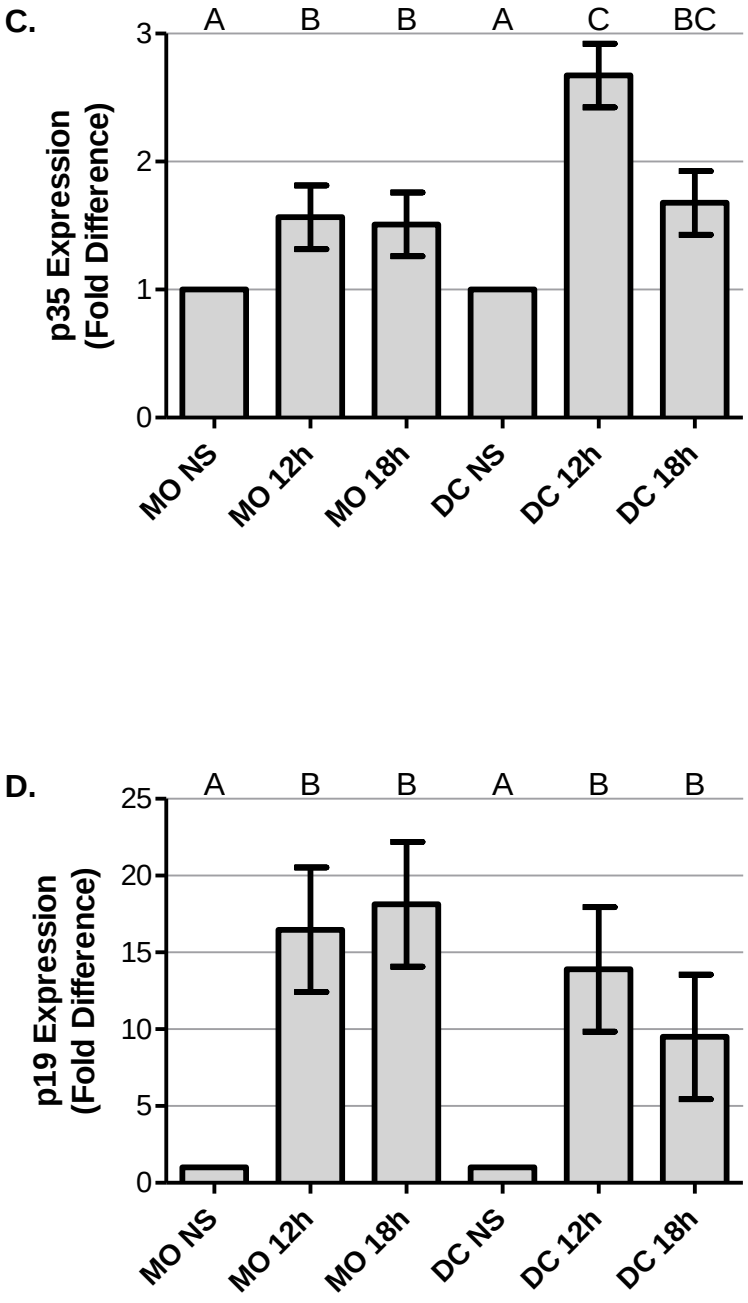
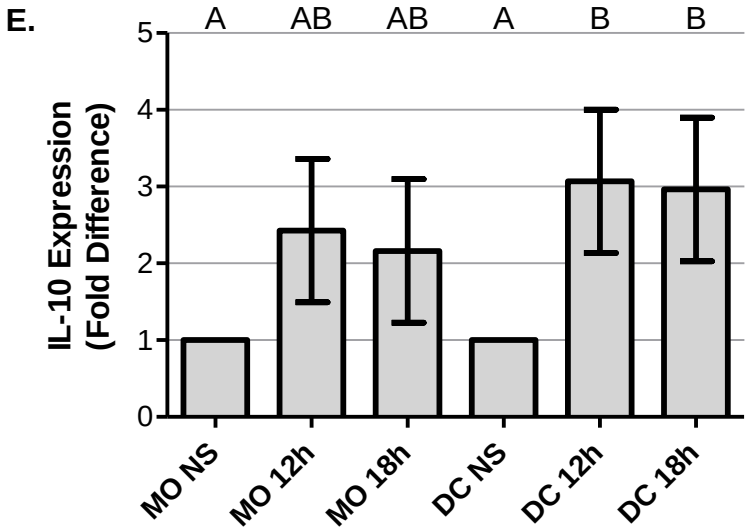


Figure 2.5. Effect of *E. coli* stimulation on equine macrophage and DC cytokine expression (continued).

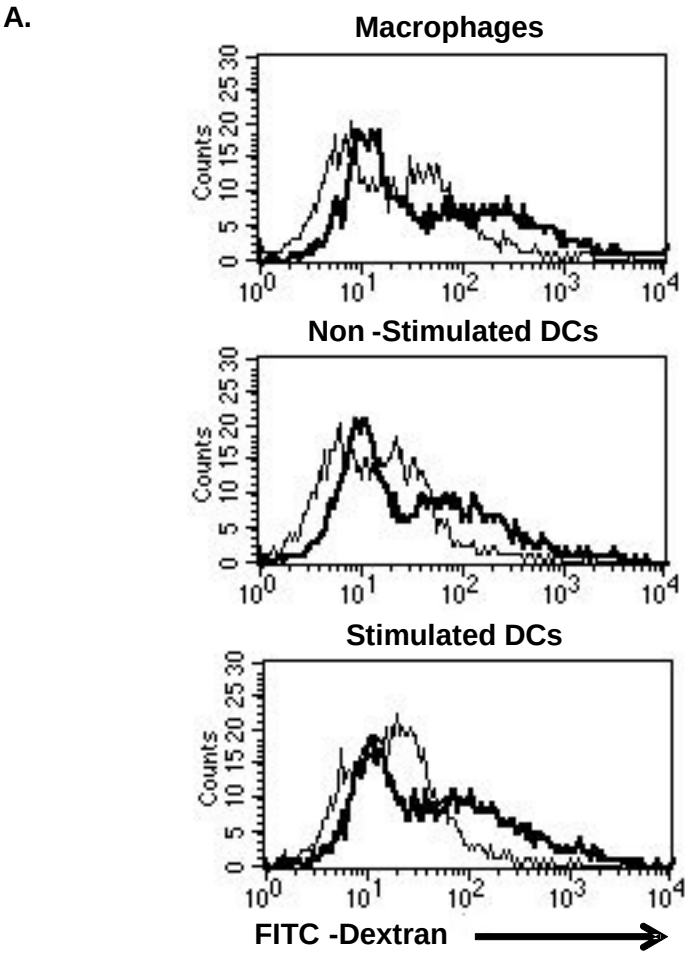


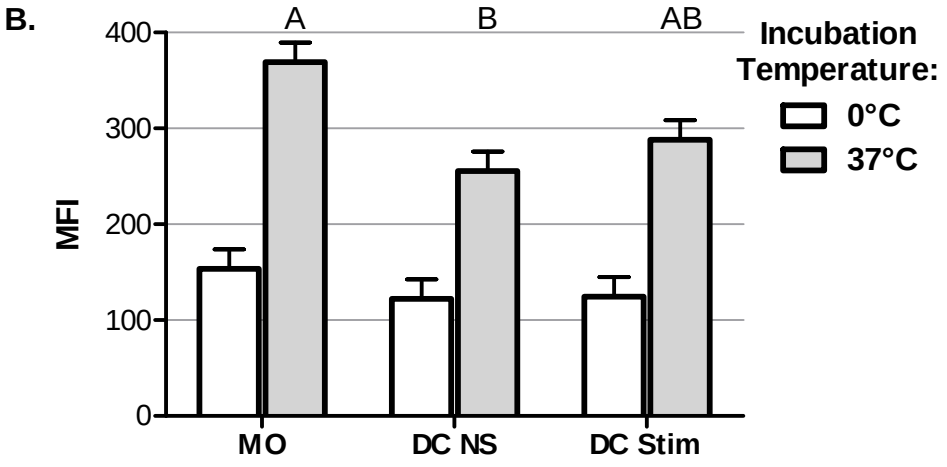
2.4.3. Effect of stimulation on endocytic capacity

All cell populations displayed a heterogeneous endocytic profile, with only a subset of cells actively taking up FITC-dextran (Figure 2.6A). Cells incubated on ice (0ºC) took up less FITC-dextran than cells incubated at 37ºC ($p < 0.0001$), and macrophages took up significantly more FITC-dextran than DCs ($p < 0.002$) (Figure 2.6B). This difference was only seen between macrophages and non-stimulated DCs when the DCs were grouped according to stimulation status. Non-stimulated and stimulated DCs endocytosed similar amounts of FITC-dextran at 37°C ($p > 0.05$).

Figure 2.6. Effect of *E.* coli stimulation on equine macrophage and DC endocytic capacity. Histograms (A) and MFI values (B) of macrophages, non-stimulated DCs, and stimulated DCs incubated at 0ºC (gray line) or 37ºC (black line) with 500 µg/mL of FITC-dextran for 30 min. Stimulated cells were incubated with UV-inactivated *E. coli* for 24 h. n = 3. MO = macrophages. DC NS = non-stimulated dendritic cells. DC Stim = stimulated dendritic cells. MFI = mean fluorescence intensity.

Figure 2.6. Effect of *E.* coli stimulation on equine macrophage and DC endocytic capacity (continued).





2.5. Discussion

A protocol for producing equine monocyte-derived dendritic cells (DCs) in complete medium with rHuGM-CSF and rEqIL-4 was originally reported by Hammond et al. (1999). Previous studies have examined the stimulation of equine DCs with single, purified molecules such as LPS, poly(I:C), and CPG-ODN (Mauel et al., 2006; Flaminio et al., 2007; Dietze et al., 2008). By incubating these cells with UV-inactivated *E. coli*, we attempted to stimulate the cells with a variety of PAMPs present on the intact bacteria, including LPS, peptidoglycan, and lipoproteins (Heumann and Roger, 2002). We have demonstrated herein that equine DCs can be activated to produce mature DCs, and we present a method for determining DC activation state. The results of these experiments have also served to further define the differences between monocyte-derived DCs and macrophages.

The expression of MHC class II was a reliable indicator of both DC cell differentiation and activation. Expression of this cell surface marker was higher in DCs than in macrophages, and both cell types increased levels of MHC class II on the cell surface following stimulation. In contrast, a similar increase in MHC class II expression was not seen in equine DCs stimulated with LPS or *Rhodococcus equi* (*R. equi*), suggesting that different stimuli on the UV-inactivated *E. coli* were necessary for this response (Dietze et al., 2008, Flaminio et al., 2009).

Quantitative RT-PCR evaluation of expression of the chemokine receptor CCR7, as well as the co-stimulatory molecules CD86 and CD40, failed to demonstrate a difference between DCs and macrophages or a marked effect of stimulation on these cells. Generally, more of these markers are thought to be expressed on DCs than on macrophages, and expression is increased upon DC stimulation (Sallusto and Lanzavecchia, 1994; Förster et al., 2008). The differences noted in our analysis were fairly small (2- to 3-fold) and of questionable biological importance or

utility for determining activation status. Upregulation of CD86 protein expression in stimulated equine DCs has been reported (Mauel et al., 2006; Flaminio et al., 2007). However, this change was very modest, which might account for the small changes we measured in mRNA expression. Additionally, failure to measure greater increases in CD86 mRNA could be due to the heterogeneous nature of the cell population, the need for additional molecular stimulants (e.g. T cell co-stimulation), or the time points at which the samples were collected.

Quantification of mRNA transcripts for cytokines revealed a strong upregulation of IL-12/IL-23p40 and IL-23p19 in stimulated macrophages and DCs. Therefore, IL-23 production is likely increased in these cells. However, it is unclear to what extent IL-12p70 protein is being produced because IL-12p35 mRNA was not upregulated but may be constitutively expressed and subject to translational regulation (Babik et al., 1999). In contrast to our results, increased expression of IL-12p35 mRNA was measured in equine DCs stimulated with LPS or *R. equi*, suggesting that IL-12p35 mRNA may be upregulated given the appropriate stimulus (Dietze et al., 2008; Flaminio et al., 2009). Interestingly, human monocyte-derived DCs stimulated with UV-inactivated *E. coli* have increased IL-23 expression but only limited production of IL-12 (Smits et al., 2004). Unfortunately, further characterization of IL-12 and IL-23 production in equine cells will require development of appropriate reagents to detect cytokine synthesis at the protein level.

Distinct TLR-mediated pathways may be involved in the expression of IL-12 or IL-23 by activated DCs. Roses et al. (2008) have shown that single stimulus of TLR-2, TLR-4, or TLR-7/8 on myeloid-derived DCs induced the production of IL-23, whereas the production of IL-12 required additional stimuli, importantly IFN-γ or multi-TLR ligands. IL-12 is a key cytokine for the development of a Th-1 type immune response, and IL-23 has been implicated in the

development of T-helper 17 (Th17) cells, which are involved in autoimmune conditions and immune responses against a variety of pathogens (Lyakh et al., 2008). If the stimulated DCs in our experiment were preferentially producing IL-23 instead of IL-12, it is possible that *E. coli* activate DCs through a limited number of PRRs. This is somewhat surprising because we chose UV-inactivated *E. coli* instead of LPS to deliberately stimulate multiple PRRS. Methods to measure IL-12 and IL-23 protein are needed to confirm if UV-inactivated *E. coli* stimulation of DCs favors IL-23 production over IL-12 and to better understand the activation signaling pathways involved.

IL-10 was measured as a control and was slightly increased in both cell types. This likely represents general activation of cytokine production in stimulated antigen presenting cells, as reported in bovine DCs stimulated with *E. coli* (Langelaar et al., 2005).

Production of TNF-α protein was greatly elevated in stimulated macrophages but was not upregulated in stimulated DCs. This contrasts with a similar study involving human monocyte-derived DCs, in which both DCs and macrophages stimulated with UV-inactivated *E. coli* increased production of TNF-α to a similar extent (Karlsson et al., 2004). However, other researchers have shown that macrophages produce more TNF-α than DCs in response to a variety of stimuli (Giacomini et al., 2001; Pietilä et al., 2005). Therefore, production of TNF-α serves to further define the differences between DCs and macrophages in our experimental system but does not indicate DC activation status.

Mannose receptor (CD206) expression decreased upon stimulation of macrophages, similar to reports in human macrophages activated with LPS (Noorman et al., 1997). The CD206 receptor binds to carbohydrate moieties of infectious agents and mediates endocytosis. The expression of this molecule is downregulated in stimulated DCs in other species and, in our

experiments, its expression was low in both stimulated and non-stimulated DCs (Apostolopoulos and McKenzie, 2001). This might indicate that the non-stimulated cells in our experiment were partially activated under normal tissue culture conditions. It is important to note, however, that the CD206 staining characteristics of equine cells were quite variable, depicted by the broad histograms of fluorescence intensity. The heterogeneous pattern of staining with this antibody against equine monocyte-derived dendritic cells has been described previously (Steinbach et al., 2005; Mauel et al., 2006). Our data suggest that CD206 is a poor marker for the activation status of equine DCs, and it reveals the heterogeneity of the DC population generated in this system. Factors that may contribute to the latter include the immune status of individual animals at the time of blood collection or inadvertent activation of DCs in tissue culture (Perrin-Cocon et al., 2008).

FITC-dextran uptake was used to measure mannose receptor (CD206)-mediated endocytic capacity (Sallusto et al., 1995). Endocytic activity was greatly reduced when the cells were incubated on ice, which suggests that measured fluorescence indicates true uptake of FITC-dextran and not simply adherence of dextran molecules to the cell surface. Endocytic activity was greater in macrophages than in DCs. However, when the dendritic cell populations were separated by activation status, this difference was only seen between macrophages and non-stimulated DCs. Stimulation of dendritic cells had no effect on FITC-dextran uptake, which provides further evidence that non-stimulated DCs are partially activated and is in agreement with the expression levels of CD206 measured in these cells. However, the histograms from these experiments indicated that the cell populations presented a heterogeneous endocytic profile; only a subset of cells endocytosed dextran molecules, which may limit the sensitivity of this assay.

Research on human DCs has revealed that the functional consequences of DC activation relate not only to the nature of the stimulus but also to the intensity, timing, and duration of that stimulus (Luft et al., 2004; Macagno et al., 2007). For example, even a very short stimulation of human monocyte-derived DCs with LPS can produce partially activated DCs with potent T cell stimulatory capacity. However, prolonged LPS stimulation is required to induce pro-inflammatory cytokine production (Macagno et al., 2006). Cytokine production is further enhanced by stimulation of multiple PRRs, and maximum production requires CD40 ligation by T cells (Schulz et al., 2000). Therefore, the quality of the stimulus may determine the degree of DC maturation. The methods used for DC generation and stimulation should be considered when comparing reported *in vitro* studies.

According to this so-called "signal integration model" of DC activation, the equine monocyte-derived DCs produced in our experiments are activated following stimulation with UV-inactivated *E. coli*. They express high levels of MHC class II, express low levels of CD206, produce large amounts of pro-inflammatory cytokines, and have low endocytic capacity. However, it appears that the non-stimulated sample may be partially activated under normal tissue culture conditions, based on the low endocytic activity of these cells and the lack of marked upregulation of CCR7, CD86, and CD40 upon stimulation. Mature DCs in our system can be distinguished from these partially activated cells based on cell surface expression of MHC class II as well as increased expression of IL-12/IL-23p40 and IL-23p19. Furthermore, equine monocyte-derived DCs can be distinguished from macrophages based on cell surface expression of MHC class II and CD206, endocytic activity, and production of TNF-α upon stimulation.

We present a method for producing and characterizing mature equine monocyte-derived DCs. We have demonstrated that DCs grown in normal tissue culture conditions may be

partially activated and can become fully mature upon stimulation with *E. coli*. The ability to measure and manipulate the activation state of equine DCs will facilitate further investigations of DC function.

2.6. Acknowledgements

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CHAPTER 3:

ACTIVATION-INDUCED FOXP3 EXPRESSION REGULATES CYTOKINE PRODUCTION IN CONVENTIONAL T CELLS STIMULATED WITH AUTOLOGOUS DENDRITIC CELLS

3.1. Summary

A defining feature of dendritic cells (DCs) is their ability to induce proliferation of autologous T cells in the absence of foreign antigen—a process termed the "autologous mixed leukocyte reaction" (AMLR). We report that equine monocyte-derived DCs, but not macrophages, are potent inducers of the AMLR. The response is contact-dependent, MHC class II-dependent, and primarily involves CD3-positive, CD4-positive, CD8-negative T cells. Upon stimulation with DCs or the mitogen Con A, a subset of the proliferating T cells expresses the regulatory T cell (Treg) transcription factor FoxP3. Although many of these FoxP3-positive T cells are capable of producing the effector cytokines IL-4 and IFN-γ, they are more likely to produce IL-10 and less likely to produce IFN-γ when compared to equivalent FoxP3-negative cells. Therefore, FoxP3 expression is an inherent component of equine T cell activation and is associated with a more immunosuppressive cytokine profile. These results confirm that FoxP3 expression in the horse, in contrast to the mouse, is regulated similarly to FOXP3 expression in humans and provide evidence that FoxP3 expression by conventional T cells may help regulate the developing immune response.

3.2. Introduction

Dendritic cells (DCs) represent a heterogeneous population of innate immune cells specialized in immune surveillance, antigen presentation, and initiation of the adaptive immune response. DCs are uniquely capable of stimulating naïve T cells and can generate a variety of effector responses, anergy, or tolerance (Mellman and Steinman, 2001). Therefore, DCs are an essential component of a successful immune response, and a detailed understanding of the interactions between DCs and T cells is relevant to many areas of immunology.

Dendritic cells take up antigen from the peripheral tissues, process it, and present it to naïve T cells in the draining lymph node. Depending on the local cytokine environment during DC-mediated T cell activation, the antigen-specific T cell can proliferate and differentiate into one of a variety of effector T cell phenotypes, including: 1) IFN-γ-producing Th1 cells that are important for immunity to intracellular pathogens and tumors; 2) IL-4-, 5-, and 13-producing Th2 cells that promote antibody production, help prevent parasitic diseases, and are involved in allergic responses; and 3) IL-17-producing Th17 cells that protect against extracellular infections (Zhu et al., 2010). DCs also play a critical role in immune regulation because they are capable of both expanding thymus-derived FoxP3+ (designated FOXP3 in humans) natural regulatory T cells (nTregs) and inducing naïve T cells to develop into induced regulatory T cells (iTregs) in the periphery (Yamazaki et al., 2003; Yamazaki et al., 2007). In turn, Tregs use a variety of mechanisms (such as production of the anti-inflammatory cytokines IL-10 and TGF-β) to keep effector responses in check and to prevent immune-mediated disease (Brusko et al., 2008).

Dendritic cells also possess the unique ability to efficiently induce proliferation of autologous T cells in the absence of exogenous antigen. This process was first demonstrated with murine DCs several decades ago and was termed the “autologous mixed leukocyte reaction”

(AMLR) (Nussenzweig and Steinman, 1980). The AMLR has since been described with human cells and is thought to represent polyclonal activation of auto-reactive T cells specific for self-antigens presented by DCs (Scheinecker et al., 1998; Amel Kashipaz et al., 2002; Chernysheva et al., 2002; Narendran et al., 2004; Barat et al., 2009). Interestingly, the AMLR displays characteristics of a normal immune response (including specificity and memory) and is reduced in a variety of human disease states (Weksler and Kozak, 1977; Indiveri et al., 1983). Furthermore, the self-reactive T cells in the AMLR demonstrate a capacity for immune suppression and increased transcription of the Treg transcription factor FOXP3—suggesting that such DC-stimulated T cells are involved in immune regulation during the normal immune response *in vivo* (Smith and Knowlton, 1979; Verhasselt et al., 2004; Jin et al., 2007).

A protocol for generating monocyte-derived DCs has been described in the horse, and the phenotype of these cells has been characterized by several groups (Hammond et al., 1999; Dietze et al., 2008; Cavatorta et al., 2009). However, relatively little is known about the ability of equine DCs to induce proliferation and differentiation of autologous T cells. Our objective in the current study was to monitor the magnitude and nature of the equine DC-induced AMLR. Previously, a detailed analysis of this interaction was limited by the use of heterogeneous cell populations and tritiated thymidine proliferation assays. We developed a method for isolating and co-culturing relatively pure populations of equine monocyte-derived DCs with autologous 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained peripheral blood T cells. We used multi-color flow cytometry to measure T cell proliferation, surface marker expression, and cytokine production.

We found that equine DCs potently induced the AMLR in the absence of foreign antigen. The AMLR-responsive T cells exhibited activation-induced FoxP3 expression—confirming that

FoxP3 is regulated similarly in horses and humans and is an inherent component of T cell activation and proliferation. Furthermore, the induced expression of FoxP3 by activated T cells was associated with a regulatory cytokine profile. This analysis contributes to our knowledge of the early events in the immune response, including concomitant regulatory mechanisms, with potential implications for immunotherapeutic strategies (Delamarre and Mellman, 2011).

3.3. Materials and methods

3.3.1. Monocyte isolation and generation of DCs

Peripheral blood was collected into 10 mL heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by jugular venipuncture from healthy adult horses according to an approved Institutional Animal Care and Use Committee (IACUC) protocol. PBMCs were isolated via 1077 Ficoll-paque (Amershan Biosciences, Piscataway, NJ) density centrifugation at 700 x g for 15 min. We modified previously described magnetic bead positive selection and plastic adherence protocols to perform monocyte isolation (Dietze et al., 2008; Steinbach et al., 2009). Briefly, 1 x 10^8 PBMCs were labeled with anti-equine CD14 monoclonal antibody (mAb) (hybridoma clone 105, kindly provided by Dr. Bettina Wagner, Cornell University, Ithaca, NY), washed with PBS, incubated with anti-mouse IgG1 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany), and positively selected over an LS column (Miltenyi Biotech) according to the manufacturer's protocol. To reduce non-specific binding, the cells were labeled in buffer containing 10% heat-inactivated autologous serum (AS), and the column was pre-treated with 500 µL of AS as described (Sung. 2008). Three million CD14+ cells were plated onto a 35 mm Petri dish (Fisher Scientific, Pittsburgh, PA) in 3 mL of Aim-V® medium (Gibco-Invitrogen, Grand Island, NY) with 10% AS and allowed to adhere for 1 h at 5% CO_2 and 37°C.

Nonadherent cells were removed by gently washing with DMEM-F12 media (Gibco-Invitrogen). DCs were produced by culturing the adherent monocyte-enriched population (approximately 2.5 x 10^6 cells) in 3 mL of DMEM-F12 complete medium (Gibco-Invitrogen) supplemented with 10% AS, 1X antibiotics/antimycotics (Gibco-Invitrogen), 10 ng/mL recombinant equine IL-4 (rEqIL-4) (kindly provided by Dr. David Horohov, University of Kentucky, Lexington, KY), and 50 ng/mL recombinant human GM-CSF (rHuGM-CSF) (R&D Systems, Minneapolis, MN) for 3 days at 5% CO_2 and 37°C. For comparison, macrophages were generated by culturing monocytes in complete medium with 10% AS, 1X antibiotics/antimycotics, and no exogenous cytokines.

3.3.2. Autologous T cell isolation and CFSE staining

On the third day of DC culture, blood was collected from the same horse, and PBMCs were isolated via 1077 Ficoll-paque density centrifugation. For negative selection of peripheral blood T cells, 1 x 10^8 PBMCs were labeled with anti-canine CD172a mAb (hybridoma clone SWC3, VMRD, Pullman, WA) and anti-human CD21 (hybridoma clone B-ly4, Becton Dickinson), washed in PBS, incubated with anti-mouse IgG1 microbeads, and eluted from an LD column according to the manufacturer's protocol (Miltenyi Biotech). The purified T cells were washed in PBS, resuspended in 0.25 μM CFSE (Sigma-Aldrich, St. Louis, MO) and incubated on a spinning rack at room temperature in the dark for 10 min (Flaminio et al., 2004). An equal volume of FBS (Gibco-Invitrogen) was added to inactivate the extracellular CFSE, and the cells were washed 3 times in PBS then resuspended in lymphocyte proliferation medium consisting of RPMI 1640 medium (Gibco-Invitrogen) enriched with 10% AS, 25 μM 2-mercaptoethanol (Sigma-Aldrich), and 1X antibiotics/antimycotics (Gibco-Invitrogen).

3.3.3. APC harvest

On day 3 of culture, non-adherent DCs and macrophages were removed by washing the wells with PBS and saved. Adherent cells were treated with 1 mL of Accumax™ (Millipore, Temecula, CA) at room temperature for 5 min and gently removed with a cell lifter. The adherent and non-adherent cells were pooled, washed in PBS, and resuspended in lymphocyte proliferation medium. Cell viability was assessed by 0.4% Trypan blue exclusion (Gibco-Invitrogen) and was consistently above 90%.

3.3.4. Autologous mixed leukocyte reaction

Purified, CFSE-stained T cells were plated in a 96-well, flat-bottom tissue culture plate (Becton Dickinson) at a concentration of 3×10^5 cells in 300 μL of lymphocyte proliferation medium. 6×10^3 DCs were added to the appropriate wells (1 DC:50 T cells). Positive control wells were treated with 5 μg/mL Con A (Sigma-Aldrich). All treatments were performed in triplicate for each horse. For the antibody-blocking experiment, 50 μL of anti-equine MHC class II mAb (CZ11 hybridoma clone 130.8 E8D9, kindly provided by Dr. Douglas Antczak, Cornell University, Ithaca, NY) or mouse anti-Parvovirus mAb (kindly provided by Dr. Colin Parrish, Cornell University, Ithaca, NY) was added to each of three wells on days 0 and 3 of culture. Samples assayed for cytokine production were treated with 40 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 μg/mL ionomycin (Sigma-Aldrich), and 10 μg/mL brefeldin A (Sigma-Aldrich) for the last 5 h of culture. Unless otherwise indicated, all samples were harvested on day 6 or 7 of culture and grown in medium containing 10% AS.

3.3.5. Transwell assay

DCs and CFSE-stained T cells were cultured in lymphocyte proliferation medium in a 96-multiwell insert system (Becton Dickinson). Because the surface area of the flat-bottom receiver plate was twice that of the 96-well tissue culture plate used for the previous DC:T cell co-cultures, 6 x 10^5 T cells and 1.2 x 10^4 DCs were plated to achieve the same cell density and DC:T cell ratio. DCs were either absent, separated from the T cells by a polyethylene terephthalate (PET) membrane with 1 μM pores, or cultured together with the T cells in the receiver plate.

3.3.6. Flow cytometry

We analyzed all samples on a FACSCalibur® flow cytometer (Becton Dickinson) equipped with argon-ion and red-diode lasers. DCs and macrophages were harvested after 3 d of culture as described, and surface marker expression was measured by incubating the cells with mAbs against equine MHC class II (CZ11 hybridoma clone 130.8 E8D9), equine CD14 (hybridoma clone 105), human CD86 (PE-conjugated hybridoma clone 2331, Becton Dickinson), or human CD206 (PE-conjugated hybridoma clone 3.29B1.10, Beckman Coulter, Fullerton, CA). Cells were washed, and the unconjugated primary mAbs were labeled with FITC-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA). T cell proliferation was quantified by measuring the percent of cells in the lymphocyte gated area that were $CFSE^{dim}$. Cell surface marker expression was analyzed by incubating cells with mAbs against equine CD3 (hybridoma clone F6G3.3, Stott Lab, UC Davis), CD4 (hybridoma clone GB61A, VMRD), CD8 (hybridoma clone HT14A, VMRD), MHC class II (CZ11 hybridoma clone 130.8 E8D9), or an isotype control (mouse anti-Parvovirus), followed

by a PE-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch). Intracellular protein expression was measured following permeabilization of cells with a saponin-based reagent (Cytofix/Cytoperm™ kit, Becton Dickinson). Cells were labeled with PE-conjugated anti-mouse/rat FoxP3 (hybridoma clone FJK-16s, eBioscience, San Diego, CA) or rat IgG2a isotype control (hybridoma clone eBR2a, eBioscience). Samples incubated with anti-mouse/rat FoxP3 antibody were also stained for expression of Alexa 647-conjugated anti-equine IL-4 (hybridoma clone 12H8, Wagner Lab, Cornell University), anti-equine IL-10 (hybridoma clone 492-2, Wagner Lab, Cornell University), or anti-bovine interferon-γ (hybridoma clone CC302, AbD Serotec, Oxford, UK).

3.3.7. Statistical analysis

For each analysis, a Shapiro-Wilk test was used to determine whether data were Gaussian and the appropriate parametric or non-parametric analysis was performed using Statistix™ 9.0 software (2008 Analytical Software, Tallahassee FL). Paired t tests were used to compare the ability of DCs and macrophages to induce the AMLR (2-sided) and to compare cytokine production between FoxP3- and FoxP3+ cells (2-sided). The appropriate parametric (paired T test) or non-parametric (Wilcoxon matched-pairs signed rank test) analysis was performed to compare surface marker expression in proliferating and non-proliferating T cells. A Spearman's rank correlation was used separately for AS and FBS samples to measure the correlation between DC dose and T cell proliferation, and a Wilcoxon rank-sum test was used to test for the effect of serum on T cell proliferation. Multiple t tests were performed to analyze the AMLR kinetics (2-sided), and separate t tests were performed for each horse to measure the effect of the transwells (2-sided) and treatment with class II MHC (1-sided to compare treated vs. untreated, 2-sided to

compare treated to baseline) or mouse anti-Parvovirus mAb on T cell proliferation. For all analyses, an overall p-value of 0.05 (2-sided) was used to indicate significance; Bonferroni adjustments to the overall p-value were used to account for multiple comparisons.

3.4. Results

3.4.1. Monocyte and T cell isolation can be obtained by magnetic sorting

To facilitate analysis of the interaction between equine monocyte-derived DCs and autologous T cells, we established a protocol for developing enriched cell populations using magnetic sorting. We isolated PBMCs from healthy adult horses by Ficoll gradient centrifugation (Figure 3.1A), and purified monocytes by positive selection and plastic adherence. Greater than 90% of cells were within the monocyte gate, and all gated cells were CD14+ (Figure 3.1B). Monocytes were cultured in complete medium supplemented with rHuGM-CSF and rEqIL-4 to generate equine monocyte-derived DCs. On day 3 of DC culture, PBMCs were again obtained from the same horse and autologous T cells were purified by negative selection. Greater than 95% of the eluted cells were detected within the lymphocyte gate, and over 95% of these cells were CD3+ (Figure 3.1C). The T cells were stained with CFSE, co-cultured with autologous DCs, and monitored for proliferation. A photomicrograph (Figure 3.2A) and a confocal image (Figure 3.2B) of co-cultured DCs and T cells are shown.

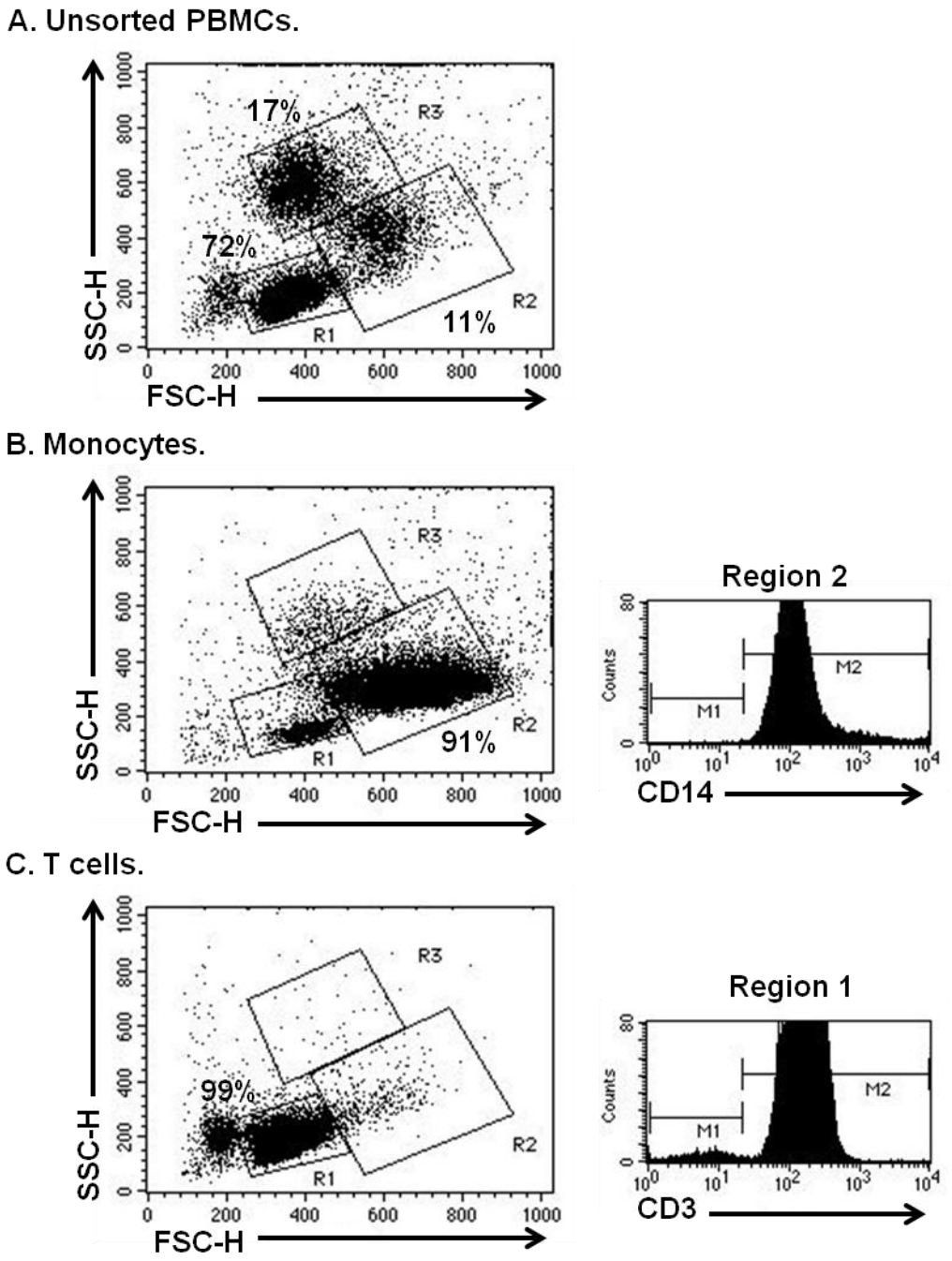


Figure 3.1. Flow cytometric dot plot and histogram analyses of peripheral blood cell sorting. Forward scatter (FSC) vs. side scatter (SSC) dot plots of pre-sorted PBMCs (A), positively selected, adherent CD14+ monocytes (B), and negatively-selected CD3+ T cells (C). Region 1 (R1) = lymphocyte gate; Region 2 (R2) = monocyte gate; Region 3 (R3) = neutrophil gate, based on cell size and granularity. Percentage values indicate percent of total cells within each gate. Histogram analyses show fluorescence intensity for CD14 positive cells (B, M2) and CD3 positive cells (C, M2); negative cells are within the M1 region.

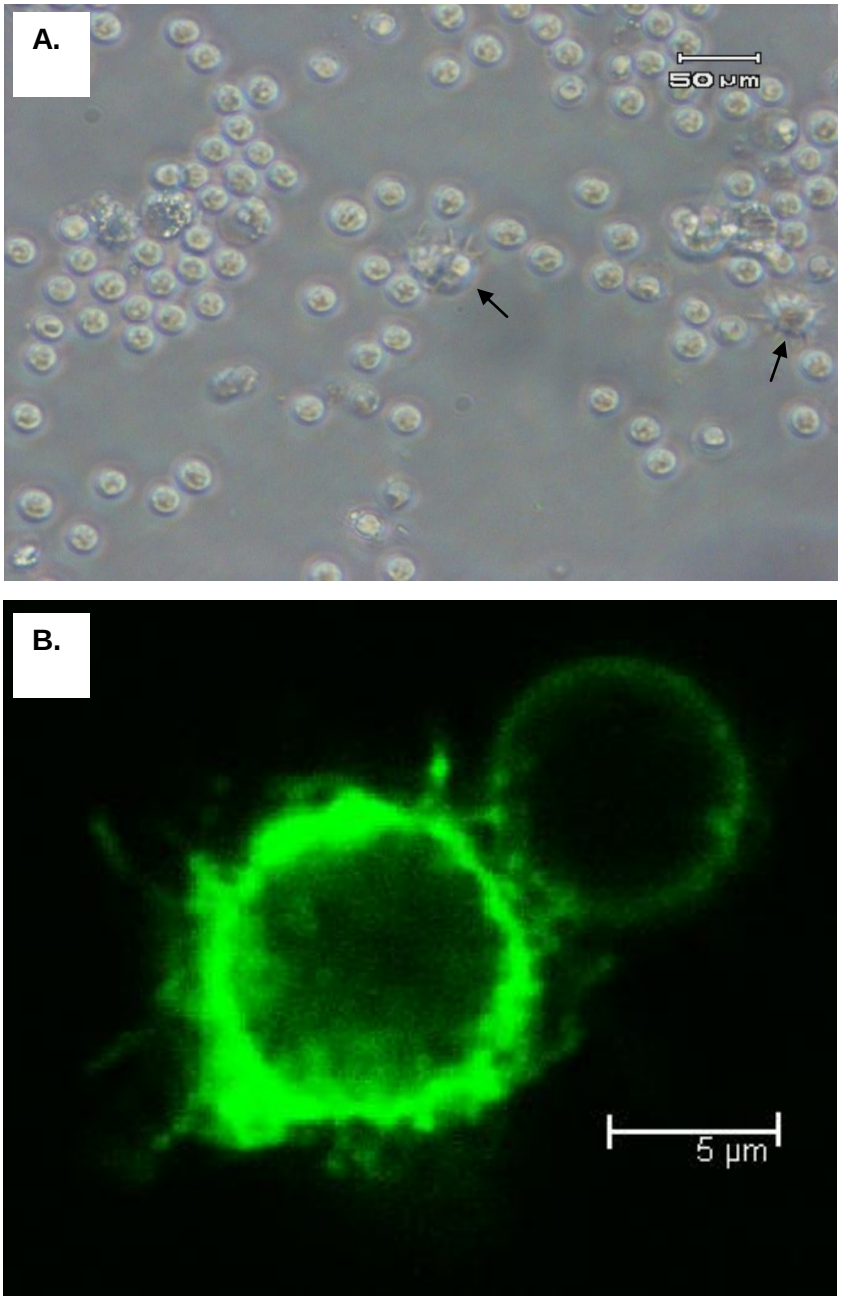


Figure 3.2. Images of co-cultured, autologous DCs and T cells. (A) Photomicrograph of DCs co-cultured with autologous T cells at a ratio of 1 DC:10 T cells. The DCs are visible as larger, more irregularly shaped cells (arrows). (B) DCs and T cells were co-cultured, labeled with FITC-conjugated anti-MHC class II antibody, and imaged by confocal microscopy. The DC is on the left and the T cell is on the right. Note the larger cell size, brighter MHC class II staining, and extensive cellular projections of the DC. These T cells were not stained with CFSE.

3.4.2. Equine DCs induce proliferation of autologous T cells in the absence of exogenous antigen

As expected, minimal proliferation was detected in cultures of non-stimulated T cells (Figure 3.3A), and a robust proliferative response was present in T cell cultures stimulated with Con A (Figure 3.3B). However, DCs induced autologous T cell proliferation in the absence of exogenous antigen (Figure 3.3C). Of note, the DC-stimulated, proliferating T cells (Figure 3.3C, cell population within 10^0 and 10^1 fluorescence) further diluted the CFSE in comparison to the Con A-stimulated, proliferating T cells (Figure 3.3B, cell population within 10^1 and 10^2 fluorescence) on day 6 of culture. The degree of T cell proliferation was highly correlated with DC concentration when the cells were grown in medium with 10% AS ($r = 0.94$, $p < 0.0001$) or 10% FBS ($r = 0.98$, $p < 0.0001$) (Figure 3.4A). Furthermore, T cell proliferation was greater in cultures grown in medium supplemented with FBS compared to AS when data were grouped by serum treatment ($p = 0.02$). When the cells were co-cultured at a ratio of 1 DC:50 T cells, a significant increase in T cell proliferation over non-stimulated controls was detected starting on day 3 of culture ($p = 0.003$) and was greatest on day 6 (Figure 3.4B). T cells cultured in the absence of DCs did not proliferate over baseline levels at any time point tested ($p > 0.045$, which is non-significant with the Bonferroni-corrected p-value).

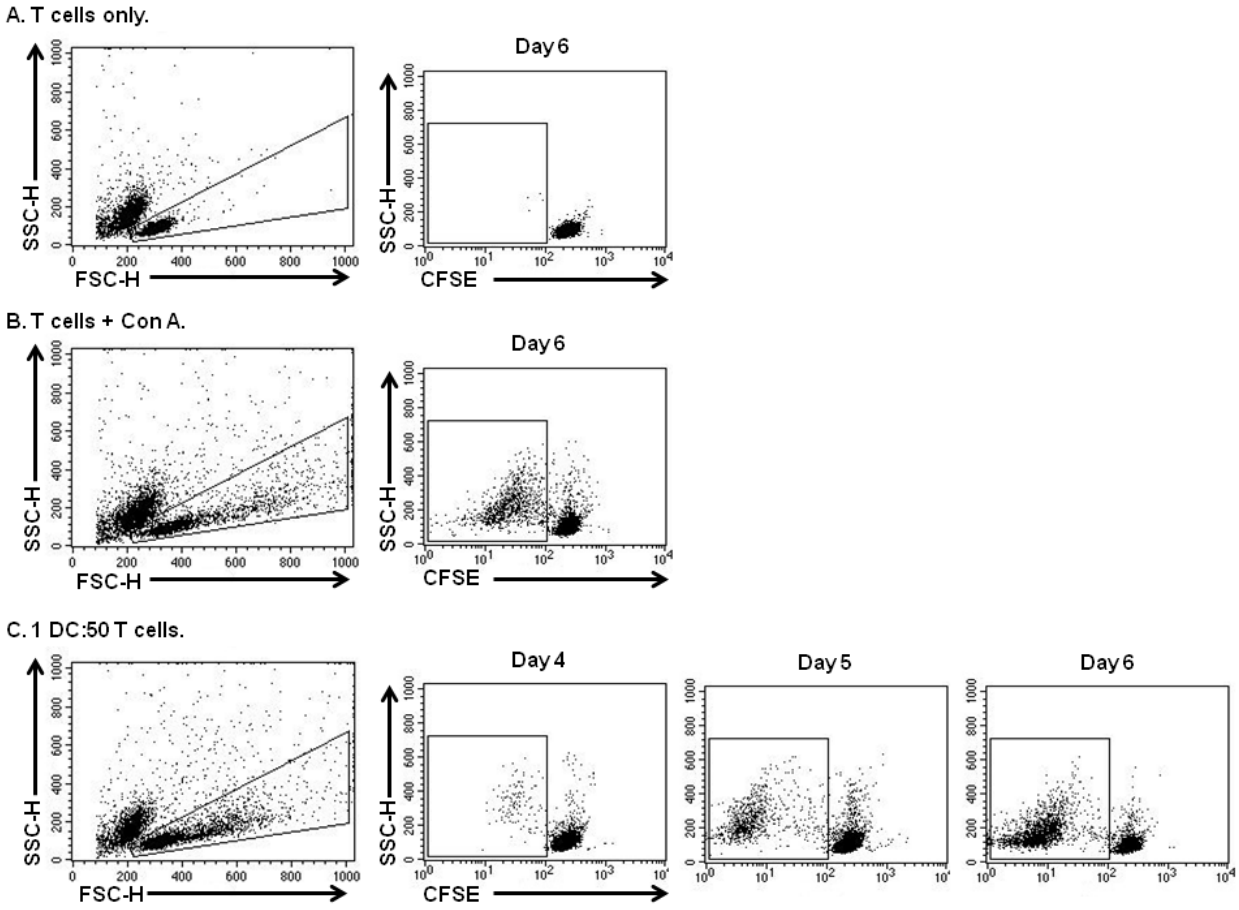


Figure 3.3. DCs induce proliferation of autologous T cells. CFSE-stained T cells were non-stimulated (A), Con A-stimulated (B), or co-cultured with DCs (C) in medium containing autologous serum. Samples were harvested on days 4 through 6 of culture for analysis by flow cytometry. Flow cytometric dot plots (FSC vs. SSC) were used to exclude dead cells by gating on lymphocytes and proliferating blast cells. Dot plots (CFSE fluorescence vs. SSC) were made for the gated lymphocytes. Non-stimulated lymphocytes were used to determine the fluorescence intensity for non-proliferating cells ($CFSE^{high}$) and proliferating cells ($CFSE^{dim}$). The percentage of proliferating cells within the $CFSE^{dim}$ region was calculated for each treatment. Note the dim fluorescence intensity of DC-stimulated proliferating T cells (C) compared to ConA-stimulated proliferating T cells (B) on day 6.

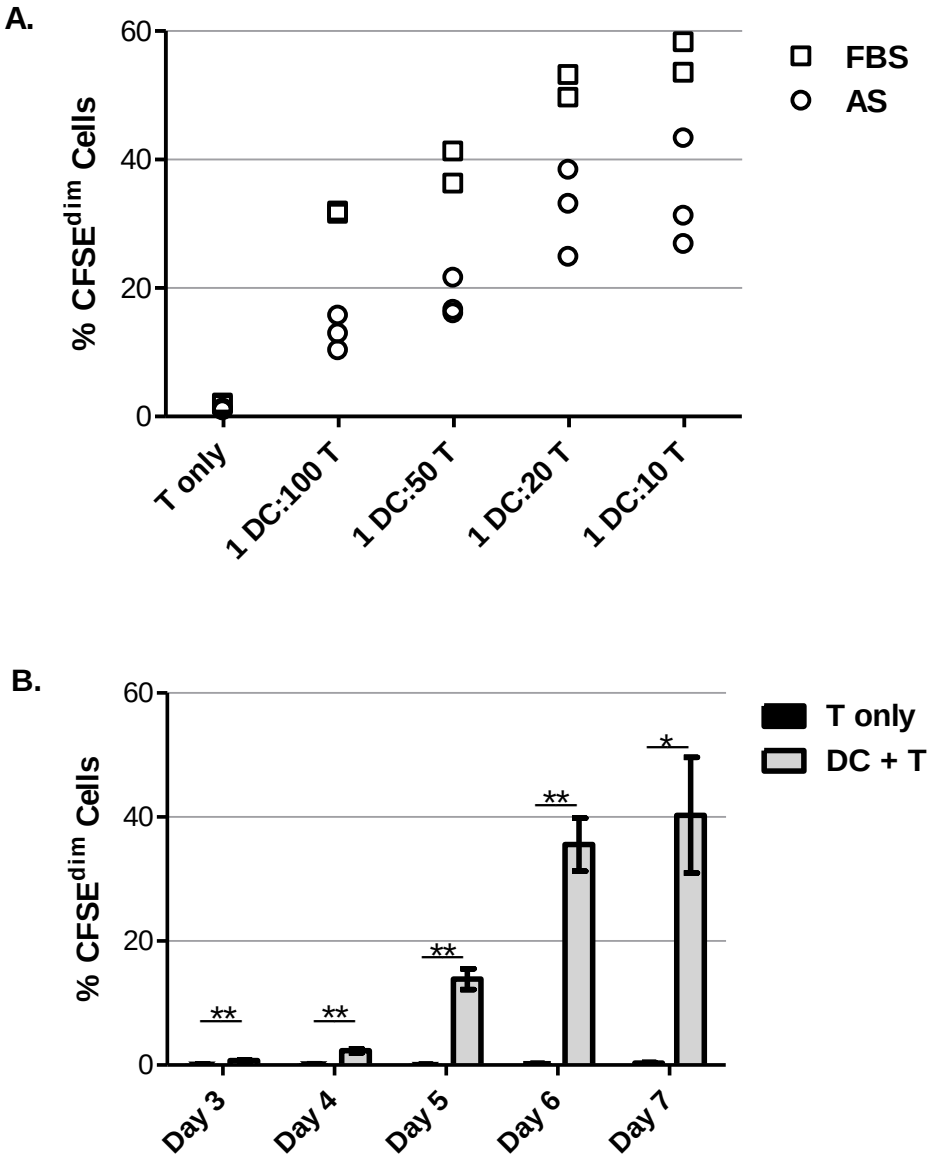


Figure 3.4. T cell proliferation correlates with DC:T cell ratio and increases with time of incubation. (A) Approximately 3 x 10^5 CFSE-stained T cells from a single horse were co-cultured alone or with varying concentrations of autologous DCs (DC:T cell ratios 1:100, 1:50, 1:20, 1:10) in culture medium containing autologous serum (AS) or fetal bovine serum (FBS). The percent of T cells within the lymphocyte gate that were proliferating ($CFSE^{dim}$) was measured after 6 days of culture by flow cytometry. Experiments were performed in triplicate (AS, open circles) or duplicate (FBS, open squares). (B) CFSE-stained T cells from a single horse were cultured alone (black bars) or with DCs (grey bars), and proliferation was monitored daily between days 3 and 7 of incubation. Each treatment was performed in triplicate. Bars represent mean and standard deviation of proliferating cells (% $CFSE^{dim}$), and values for the two treatment groups were compared. * $p < 0.05$. ** $p < 0.01$.

3.4.3. DCs stimulate T cells in a contact-dependent, MHC class II-dependent manner

We attempted to determine the nature of the DC stimulatory signal in the AMLR because some cells (such as memory T cells) can undergo homeostatic proliferation in the presence of DC-derived cytokines (Geginat et al., 2001). We used a transwell system to prevent direct contact between DCs and T cells while permitting diffusion of cytokines between the two cell types. When direct contact with DCs was prevented by a permeable membrane, T cell proliferation was reduced to background levels (both $p < 0.005$) (Figure 3.5A). Preventing T cell receptor (TCR) recognition of MHC class II-peptide complexes with anti-MHC class II blocking antibody decreased T cell proliferation significantly (all $p \leq 0.02$) in all horses tested but not completely to baseline levels in 1 of the 3 horses ($p = 0.045$, which is non-significant with the Bonferroni-corrected p-value) (Figure 3.5B). In contrast, adding an irrelevant monoclonal antibody (mouse anti-Parvovirus) had no effect on T cell proliferation in either of the two horses tested ($p > 0.05$) (Figure 3.5C).

Figure 3.5. T cell proliferation requires DC contact and is MHC class II-dependent. (A) Using a transwell system, CFSE-stained T cells were cultured in the bottom chamber either alone (T only), combined with DCs (DC + T together), or separated from DCs by a permeable membrane (DC + T separate). Cells from 2 different horses were cultured in medium containing autologous serum for 7 days, in triplicate. The samples with T cells alone were cultured as a control and were not included in the statistical analysis. (B) CFSE-stained T cells were cultured alone (T only), with DCs (DC + T), or with DCs plus anti-equine MHC class II monoclonal antibody (DC + T + MHCII mAb). Cells from 3 different horses were cultured in medium containing autologous serum for 5 to 7 days, in triplicate. (C) CFSE-stained T cells were cultured with DCs (DC + T), with DCs plus anti-equine MHC class II monoclonal antibody (DC + T + MHCII mAb), or with DCs plus an irrelevant antibody, mouse anti-Parvovirus (DC + T + MxP mAb). Cells from 2 different horses were cultured in medium containing autologous serum for 5 to 7 days, in triplicate. Data from horse #215 are from the same experiment in parts (B) and (C), but data from horse #70 are from 2 independent experiments (the effect of MxP mAb was not tested in the first experiment). For all graphs, bars represent mean and standard deviation of the percentage of cells within the lymphocyte gate that were proliferating ($CFSE^{dim}$). * $p < 0.025$. ** $p < 0.005$. "a" indicates mAb treated group is not equal to T only ($p < 0.02$).

Figure 3.5. T cell proliferation requires DC contact and is MHC class II-dependent (continued).

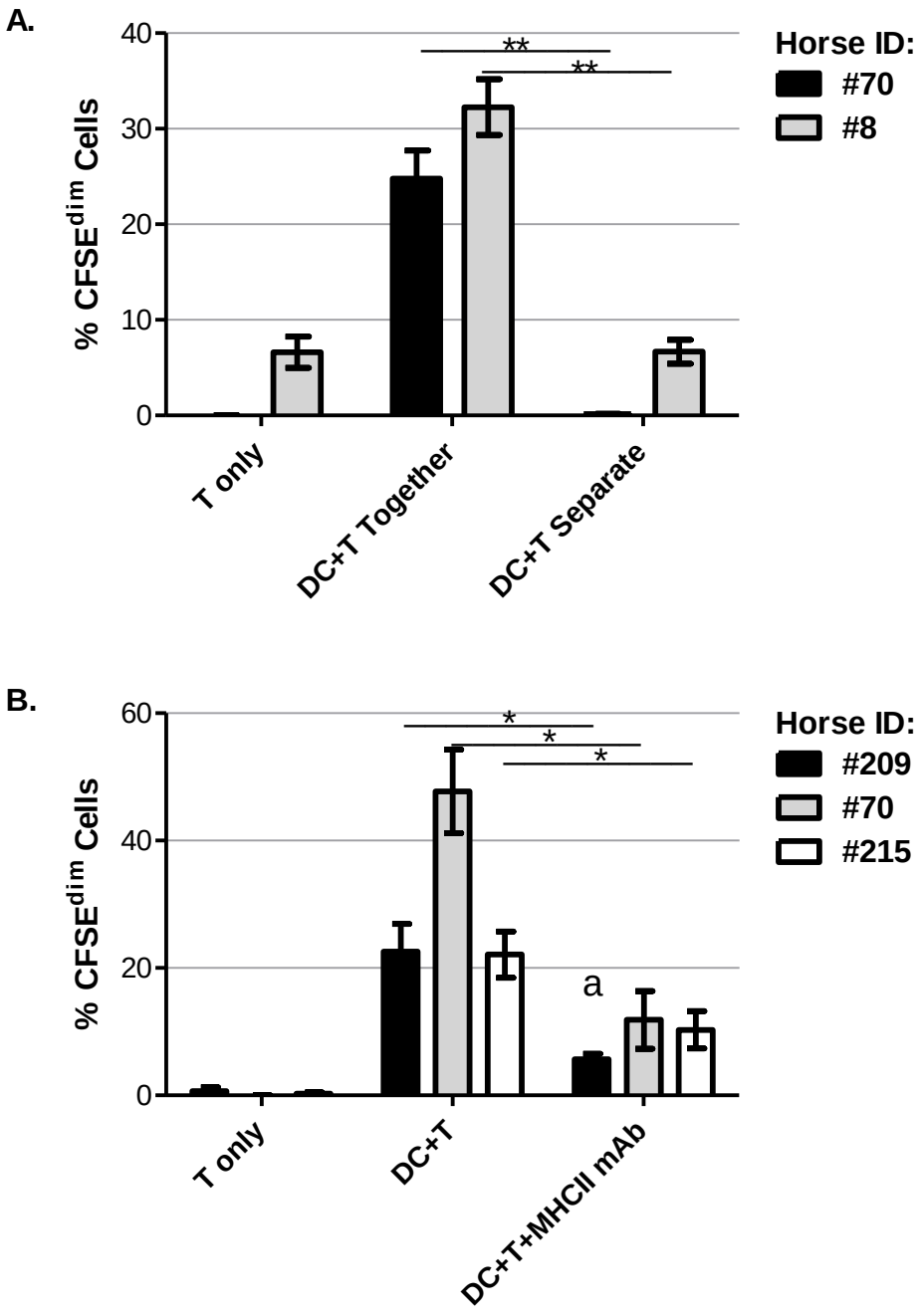
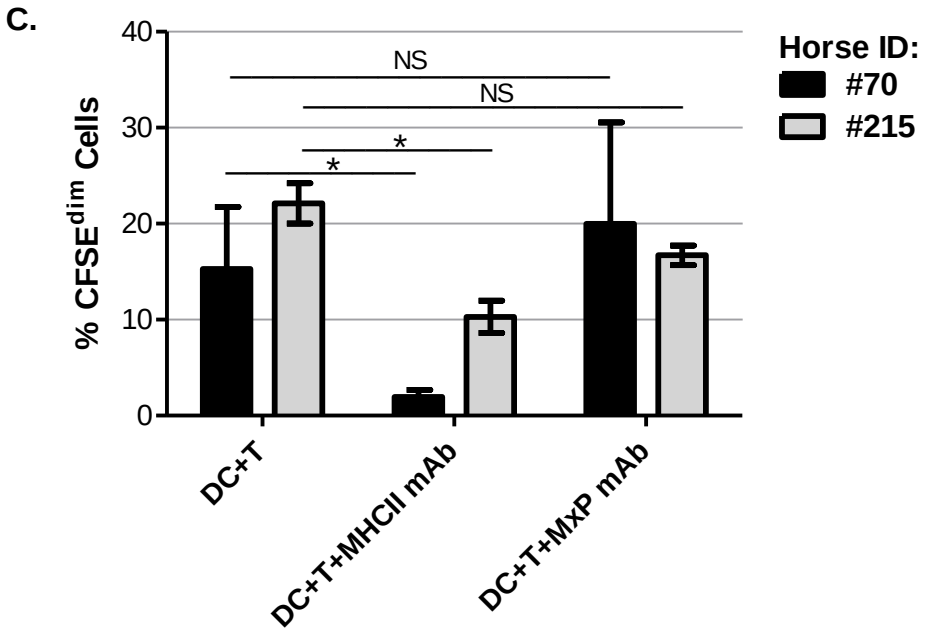


Figure 3.5. T cell proliferation requires DC contact and is MHC class II-dependent (continued).



3.4.4. DCs are more potent inducers of the AMLR compared to macrophages

The ability to efficiently induce the AMLR is a defining characteristic of DCs (Nussenzweig and Steinman, 1980; Freudenthal and Steinman, 1990; Scheinecker et al., 1998). Therefore, we wanted to compare the ability of equine monocyte-derived DCs and macrophages to induce proliferation of autologous T cells in the absence of foreign antigen. Flow cytometric phenotyping revealed that DCs were MHC class $II^{high}CD14^{low}$ cells (Figure 3.6A). Most DCs were CD86-$CD206^{high}$, consistent with an immature phenotype—although a subset (approximately 10 to 30%) of cells were $CD206^{low}$CD86+ mature DCs. The $CD206^{low}$CD86+ DCs were less refractile than the immature DCs, as indicated on FSC vs. SSC dot plots (Figure 3.6B). In contrast, macrophages were MHC class $II^{low}CD14^{high}CD86^{low}CD206^{low}$ cells and consistently included a small population (less than 5%) of cells with increased expression of MHC class II. As expected from these phenotypic profiles, equine DCs were more potent inducers of T cell proliferation in the AMLR compared to macrophages ($p \leq 0.002$) (Figure 3.6C).

Figure 3.6. DCs are more efficient inducers of the AMLR compared to macrophages. (A) DCs and macrophages (MOs) were harvested after 3 days of culture in medium containing autologous serum and stained for surface expression of MHC class II, CD14, CD86, or CD206. Histogram analyses were made to compare expression of each surface marker between DCs and macrophages. Shaded region, isotype control. Wide line, DCs. Thin line, macrophages. Data are representative of 4 independent experiments. (B) Additional gating was performed to analyze forward scatter (FSC) and side scatter (SSC) properties of mature ($CD206^{low}CD86+$) and immature ($CD206^{high}CD86-$) cells. Data are representative of 4 independent experiments. (C) CFSE-stained T cells from 5 different horses (one horse was used twice) were cultured with DCs or MOs. Cells were cultured in medium containing autologous serum for 6 to 7 days, in triplicate. Each data point represents the mean percentage of cells within the lymphocyte gate that were proliferating (% $CFSE^{dim}$) for each horse. Values for each treatment group were compared. Lines indicate DC and MO data are from the same horse. * $p \leq 0.002$.

Figure 3.6. DCs are more efficient inducers of the AMLR compared to macrophages (continued).

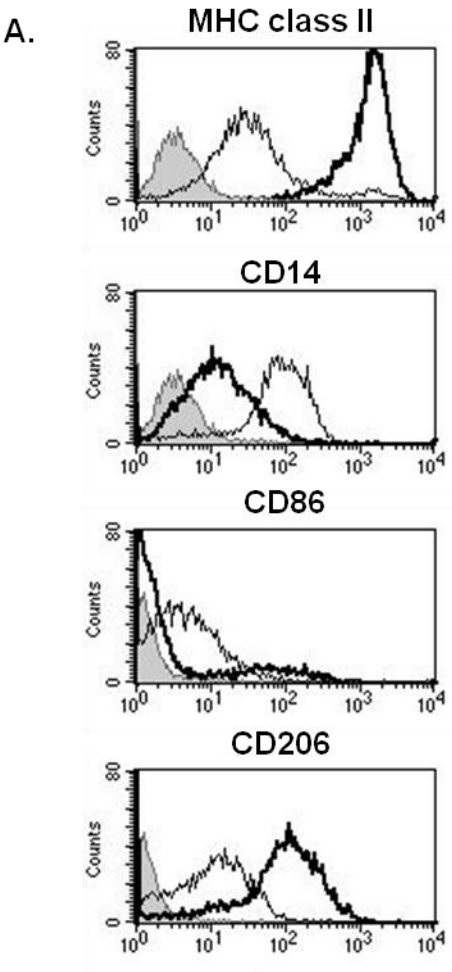


Figure 3.6. DCs are more efficient inducers of the AMLR compared to macrophages (continued).

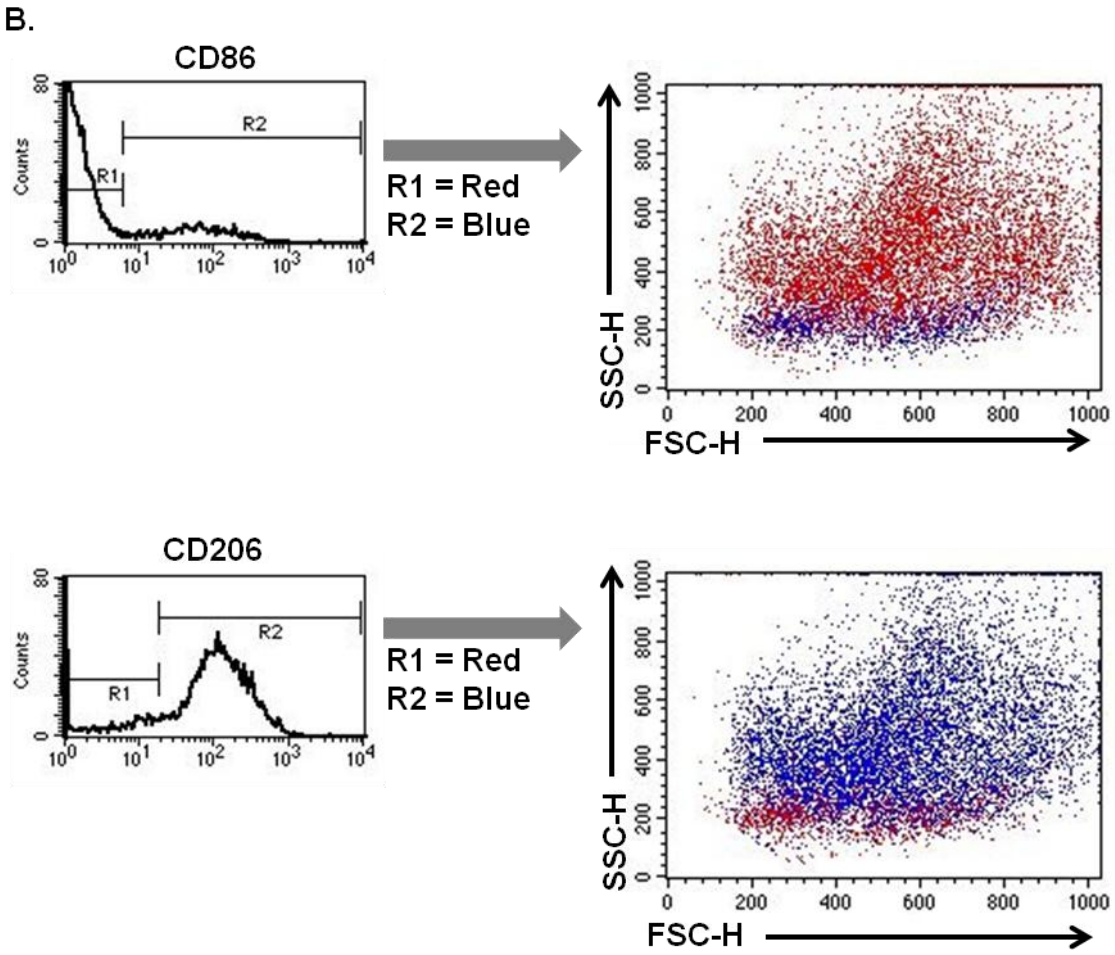
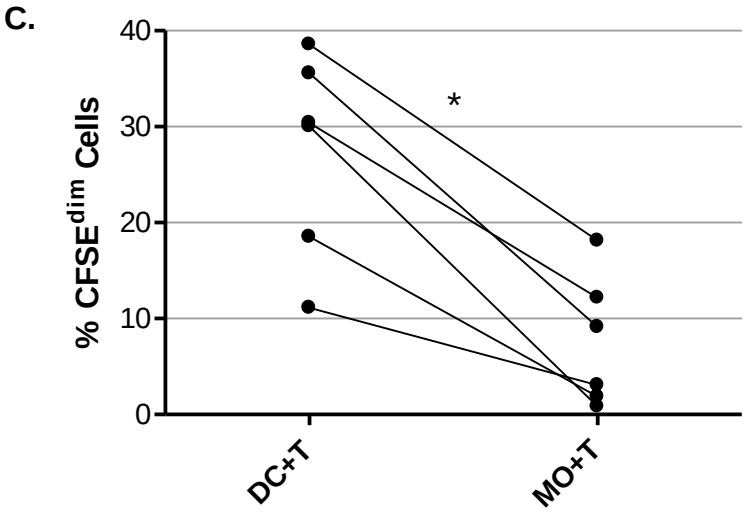


Figure 3.6. DCs are more efficient inducers of the AMLR compared to macrophages (continued).



3.4.5. Proliferating T cells in the AMLR are primarily CD3+CD4+CD8- T cells

CFSE-stained, DC-stimulated T cells were labeled with surface markers to characterize the proliferating T cell population after 6 days of culture. As expected, virtually all cells in the lymphocyte gate were CD3+ T cells (Figure 3.7A). The proliferating $CFSE^{dim}$ cells were primarily CD4+CD8- T cells. The T cells were positive for MHC class II, a feature demonstrated previously for adult equine T cells (Lunn et al., 1993). Expression of CD3 was equivalent in proliferating and non-proliferating cells ($p > 0.05$), but CD4 and MHC class II expression were increased in proliferating cells ($p \leq 0.0005$ and $p \leq 0.008$, respectively), consistent with an activated phenotype (Figure 3.7B) (Bendali-Ahcene et al., 1997).

3.4.6. A subset of DC-stimulated T cells expresses FoxP3

Murine DCs induce expansion of nTregs and differentiation of naïve T cells to become FoxP3+ iTregs by presenting self-antigens in the periphery (Morel and Turner, 2011). Because the T cells in the equine AMLR appeared to be responding to DCs in the absence of exogenous antigen, we measured their expression of the Treg transcription factor FoxP3. A subset of the proliferating T cells (5 to 15%) expressed FoxP3 after 6 days of culture with autologous DCs (Figure 3.7C).

Figure 3.7. AMLR induces proliferation of CD4+ T cells, including a subpopulation of FoxP3+ cells. CFSE-stained T cells were cultured alone or with DCs in medium containing autologous serum. Cells were harvested after 6 days of incubation and (A) stained for surface expression of CD3, CD4, CD8, or MHC class II. (B) Data from multiple experiments (n = 13 for CD3, n = 13 for CD4, n = 8 for MHC class II) were analyzed to compare surface marker expression between proliferating (upper left quadrant from Figure A) and non-proliferating (upper right quadrant from Figure A) cells. Lines indicate data are from the same sample. (C) Other cells were harvested after 6 days of incubation, permeabilized and stained for intracellular levels of FoxP3 or a negative isotype control. Data are representative of 5 independent experiments. * $p \leq 0.008$. MFI, mean fluorescence intensity.

Figure 3.7. AMLR induces proliferation of CD4+ T cells, including a subpopulation of FoxP3+ cells (continued).

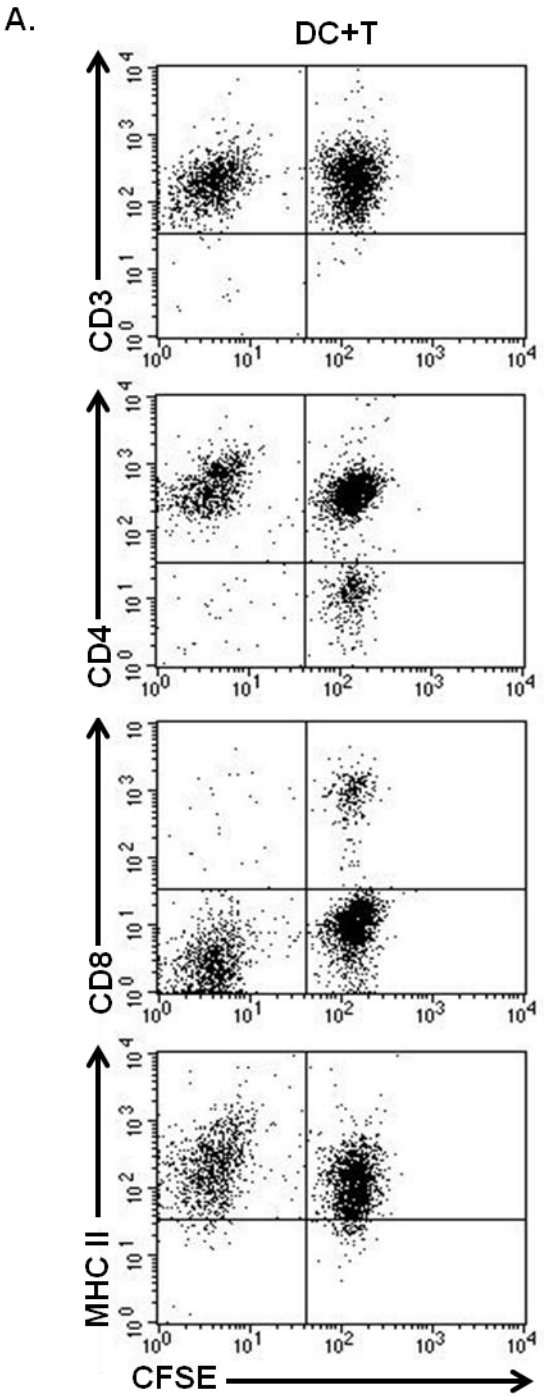


Figure 3.7. AMLR induces proliferation of CD4+ T cells, including a subpopulation of FoxP3+ cells (continued).

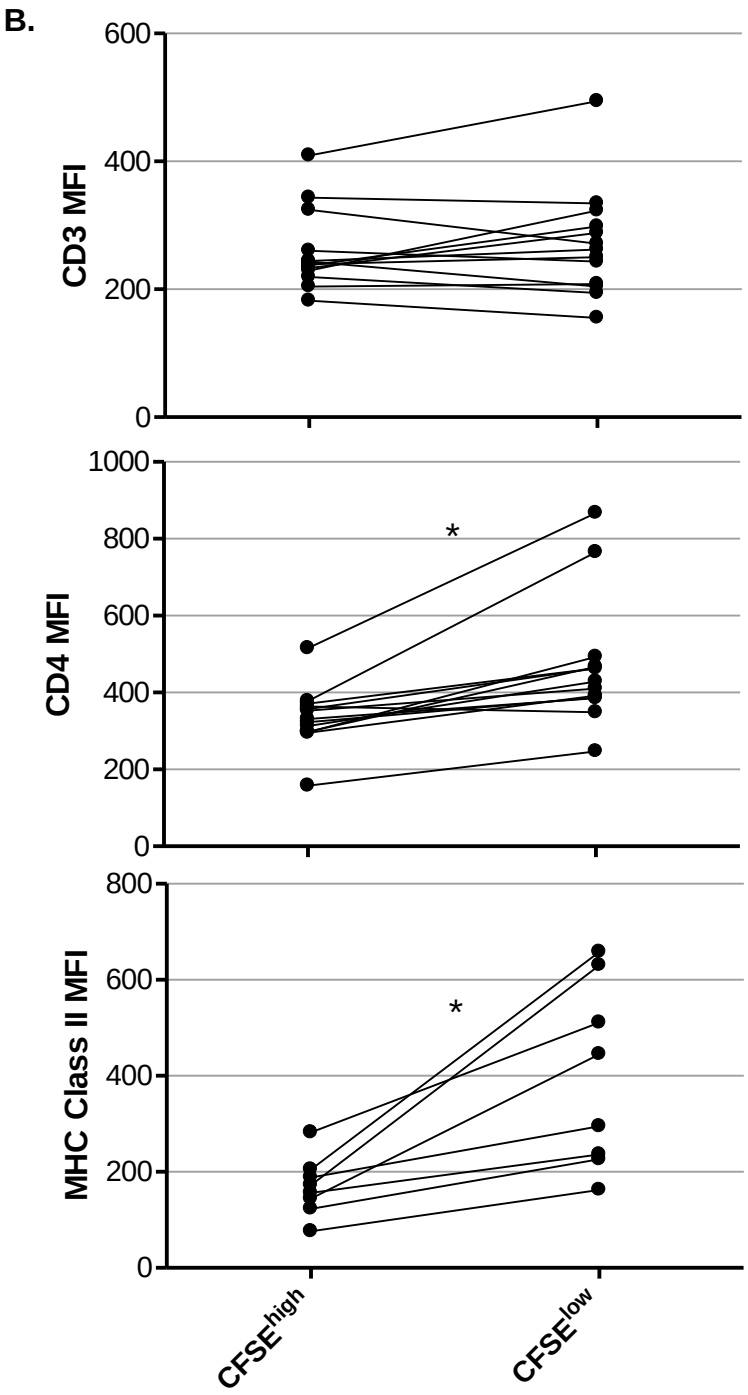
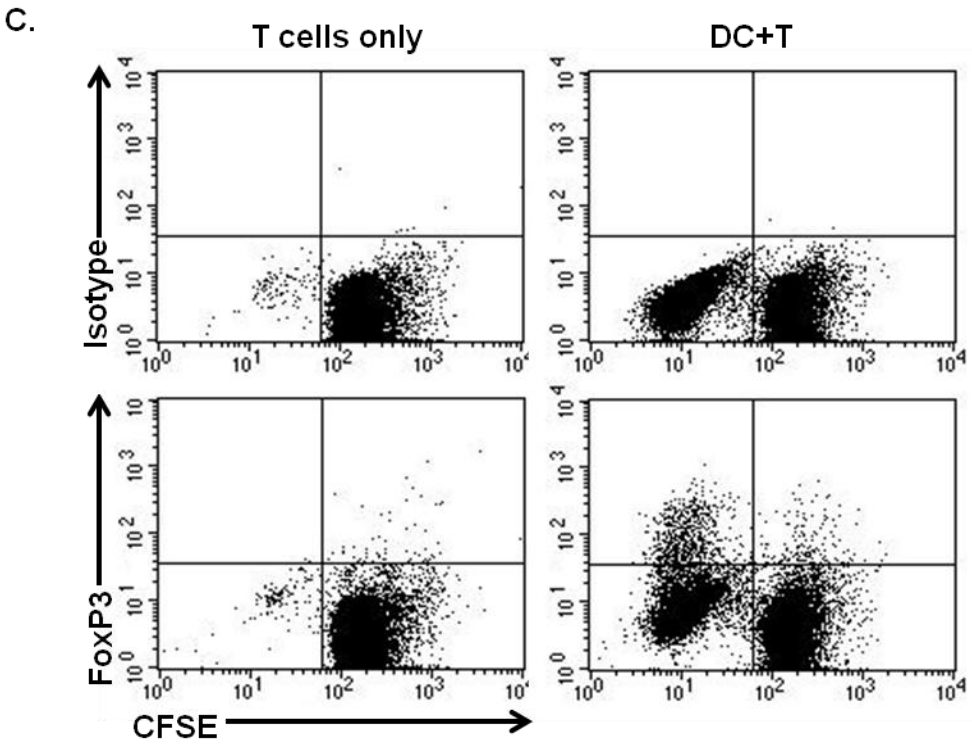


Figure 3.7. AMLR induces proliferation of CD4+ T cells, including a subpopulation of FoxP3+ cells (continued).



3.4.7. Proliferating FoxP3+ cells can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ

Although initial experiments in mice lead researchers to believe that FoxP3 is a Treg-specific transcription factor, accumulating evidence suggests that FOXP3 is also expressed transiently in activated human conventional T cells (Valencic et al., 2007). Additionally, Con A stimulation increases FoxP3 expression in equine peripheral blood T cells (Robbin et al., 2011). Therefore, acquisition of FoxP3 expression in DC-stimulated equine T cells might simply be a transient marker of cellular activation and might not be associated with a regulatory phenotype. To investigate this question further, we decided to measure cytokine production as an indicator of T cell function. Preliminary experiments indicated that PMA and ionomycin were required to induce cytokine production in DC-stimulated T cells (Figure 3.8). Therefore, we treated DC-stimulated, CFSE-stained T cells with PMA plus ionomycin and compared cytokine production by $CFSE^{dim}$FoxP3+ cells with that of $CFSE^{dim}$FoxP3- cells (Figure 3.9A.). We found that approximately equivalent subpopulations of FoxP3+ T cells were capable of producing the effector cytokines IFN-γ and IL-4 as well as the regulatory cytokine IL-10 (Figure 3.9B). However, when compared to the FoxP3- cells, more of the FoxP3+ cells produced IL-10 ($p = 0.015$) and fewer produced IFN-γ ($p = 0.013$) (Figure 3.9C). Both cell populations contained statistically equivalent numbers of IL-4-competent cells ($p = 0.095$). Approximately 60% of the FoxP3+ and FoxP3- cells did not express detectable levels of IFN-γ, IL-4, or IL-10.

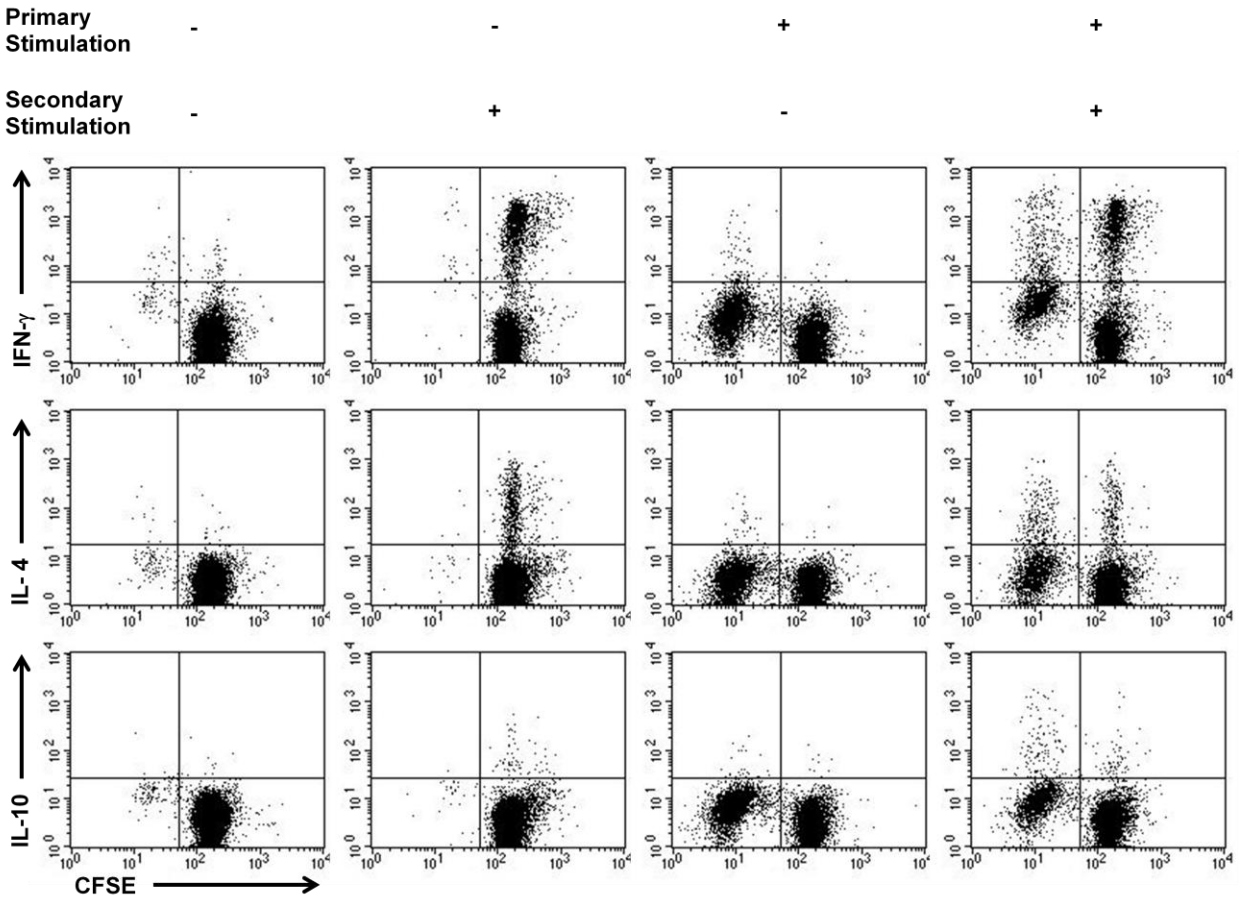


Figure 3.8. Secondary stimulation with PMA and ionomycin is required to measure cytokine production in AMLR-responsive T cells. CFSE-stained T cells were cultured alone or with DCs (primary stimulation) in medium containing autologous serum for 6 days. Cells were treated with brefeldin A with or without PMA and ionomycin (secondary stimulation) for the last 5 hours of culture. Cells were harvested, permeabilized, and stained for cytokine production (IFN-γ, IL-4, or IL-10). Data are representative of 3 independent experiments.

Figure 3.9. AMLR reactive FoxP3+ lymphocytes can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ. CFSE-stained T cells from 5 different horses were cultured alone or with DCs in medium containing autologous serum. Cells were cultured for 6 days and treated with PMA, ionomycin and brefeldin A for the last 5 hours of culture. Cells were harvested, permeabilized, and stained for FoxP3 expression and cytokine production (IFN-γ, IL-4, or IL-10). (A) Cells within the lymphocyte gate were gated for proliferation ($CFSE^{dim}$), and cytokine profiles were measured for FoxP3+ and FoxP3- cells. (B) Cytokine production by FoxP3+ cells. Bars represent mean percentage of cytokine-producing cells. (C) Cytokine production was compared between FoxP3+ and FoxP3- cells. Lines indicate FoxP3+ and FoxP3- data are from the same horse. * $p \leq 0.015$.

Figure 3.9. AMLR reactive FoxP3+ lymphocytes can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ (continued).

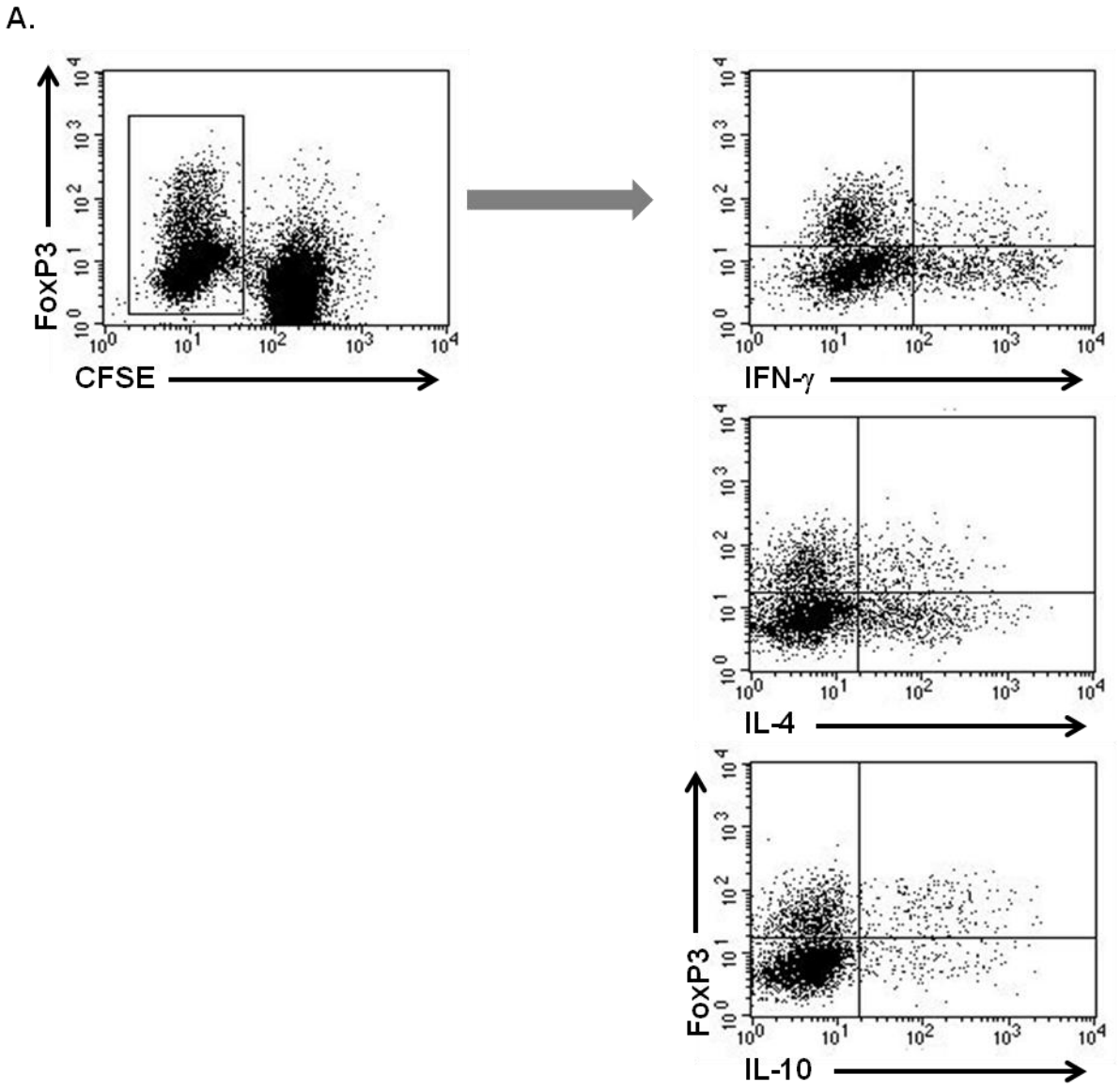


Figure 3.9. AMLR reactive FoxP3+ lymphocytes can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ (continued).

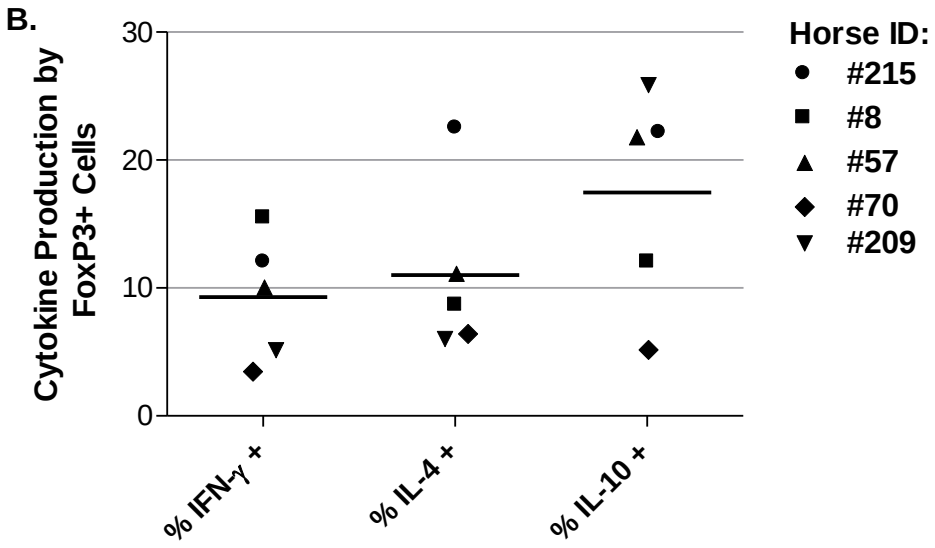
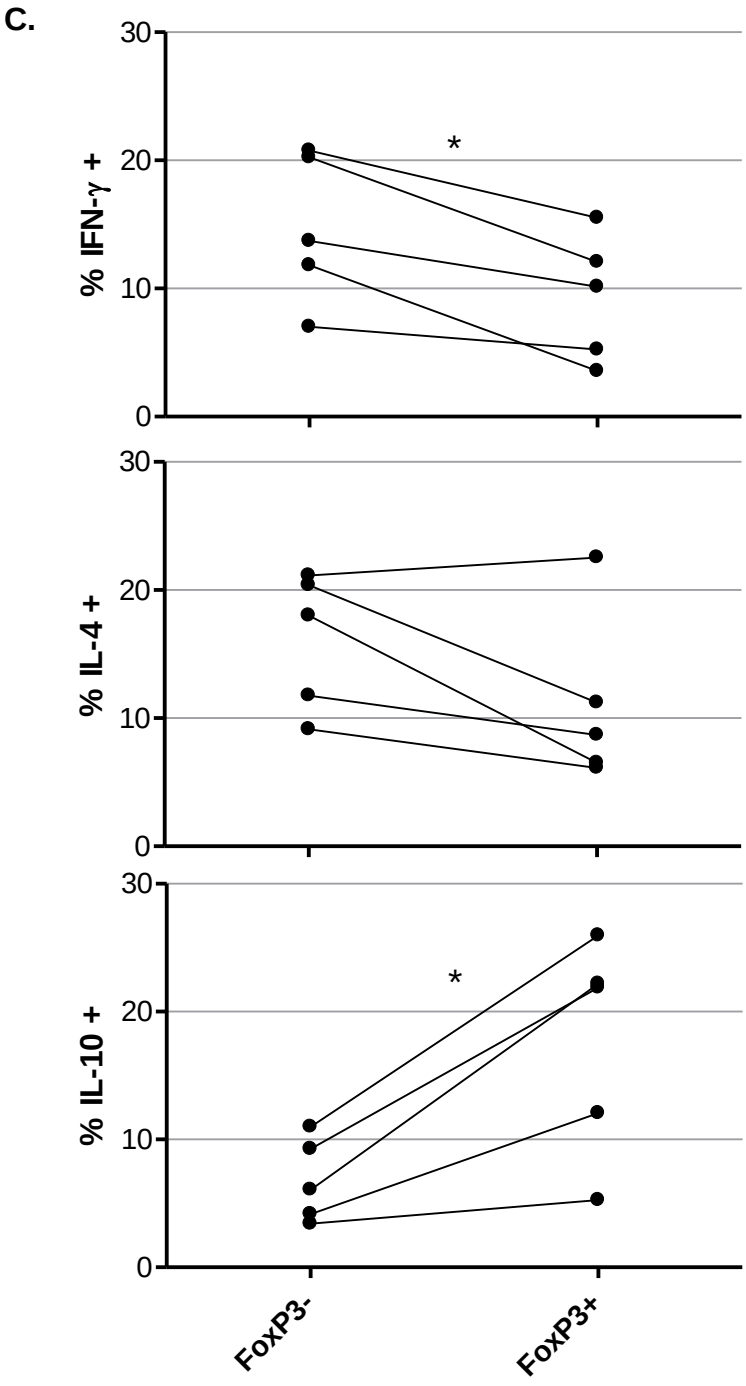


Figure 3.9. AMLR reactive FoxP3+ lymphocytes can produce effector cytokines but more of these cells produce IL-10 and fewer produce IFN-γ (continued).



3.4.8. Cytokine production by FoxP3+ vs. FoxP3- cells is similar following stimulation with DCs or Con A

Dendritic cells in humans and mice can adopt a tolerogenic phenotype and promote the differentiation and expansion of Treg populations (Kushwah and Hu, 2011; Zanoni and Granucci, 2011). Therefore, we wondered whether the regulatory cytokine profile detected in DC-stimulated FoxP3+ T cells was triggered by DC-derived signals or whether it was an inherent component of T cell activation and proliferation in the horse. Dendritic cells in humans and mice can adopt a tolerogenic phenotype and promote the differentiation and expansion of Treg populations (31, 32). Therefore, we wondered whether the regulatory cytokine profile detected in DC-stimulated FoxP3+ T cells was triggered by DC-derived signals or whether it was an inherent component of T cell activation and proliferation in the horse. We compared cytokine production by $CFSE^{dim}$FoxP3+ cells with that of $CFSE^{dim}$FoxP3- cells in DC- and Con A-stimulated T cell cultures. Cells were cultured for 6 days and stimulated with PMA and ionomycin prior to analysis. Similar to the DC-stimulated samples, more of the Con A-stimulated FoxP3+ cells produced IL-10 and fewer produced IFN-γ (Figure 3.10).

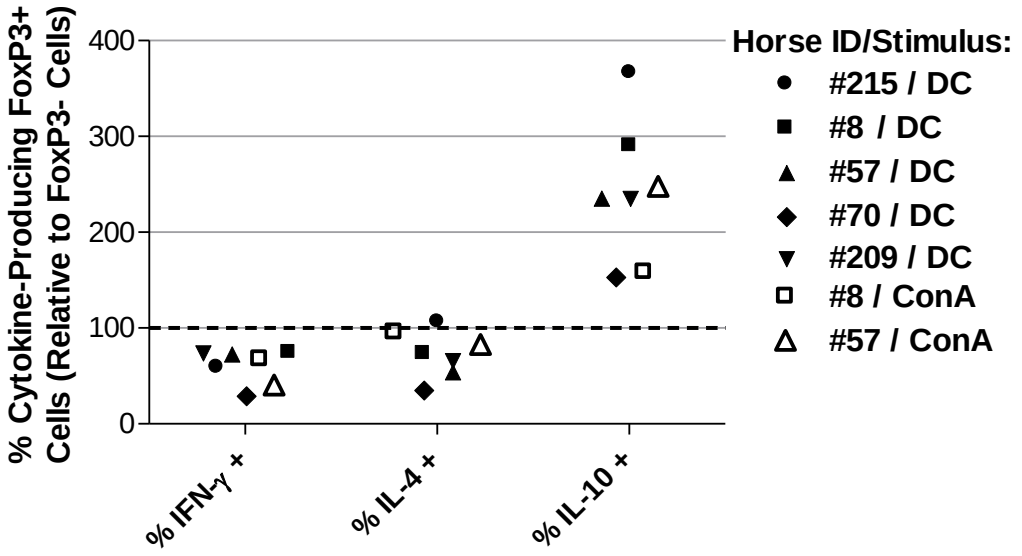


Figure 3.10. FoxP3+ and FoxP3- cytokine profiles are similar in DC- and Concanavalin A-stimulated T cells. CFSE-stained T cells from 5 different horses were cultured with DCs (5 horses) or Concanavalin A (2/5 horses) in medium containing autologous serum. Cells were cultured for 6 days and treated with PMA, ionomycin and brefeldin A for the last 5 hours of culture. Cells were harvested, permeabilized, and stained for FoxP3 expression and cytokine production (IFN-γ, IL-4, or IL-10). Cells within the lymphocyte gate were gated for proliferation ($CFSE^{dim}$), and cytokine production by FoxP3+ cells relative to FoxP3- cells was calculated. Relative expression equals 100 x (% of FoxP3+ cells producing cytokine ÷ % of FoxP3- cells producing cytokine). Values below 100% (dotted line) indicate fewer cytokine-producing FoxP3+ cells compared to FoxP3- cells. Values above 100% indicate more cytokine-producing FoxP3+ cells compared to FoxP3- cells. Filled shapes indicate DC stimulation. Open shapes indicate ConA stimulation.

3.5. Discussion

In this study, we showed that equine monocyte-derived DCs, but not macrophages, were capable of stimulating the AMLR in the absence of foreign antigen. To our knowledge, this is the first description of the AMLR in the horse. As in humans and mice, the equine AMLR was contact- and MHC class II-dependent and primarily involved CD3+CD4+CD8- T cells (Innes et al., 1989; Kawamura et al., 1991). We have also shown for the first time that DC-responsive T cells express increased levels of CD4 and MHC class II molecules, consistent with an activated phenotype.

Equine DCs express high levels of MHC class II, contain a subpopulation of cells with a mature flow cytometric phenotype, and more potently induce the AMLR compared to macrophages (Cavatorta et al., 2009). This confirms the superior antigen-presenting and co-stimulatory capacity of equine DCs, because the AMLR requires DC expression of CD86 and MHC class II (Scheinecker et al., 1998). The low levels of T cell proliferation measured in the macrophage co-cultures might be due to the presence of small numbers of contaminating DCs consistently detected as MHC class II^{high} cells by flow cytometric phenotyping. These could be unsorted DCs from peripheral blood or spontaneously differentiated, monocyte-derived DCs (Ho et al., 2002). The discrepancy in CD206 expression by macrophages and DCs between this experiment and that described in Chapter 2 is likely due to differences in cell purity and culture duration; CD206 expression by cultured equine DCs appears to decrease with time.

The demonstration that T cell proliferation in the AMLR was contact- and MHC class II-dependent confirms that these cells were not exclusively responding to DC-derived cytokines. Instead, the T cells responded through the TCR to antigens presented by DCs. T cell proliferation was highly correlated with DC dose and, at a DC:T cell ratio of 1:50, was detectable

by day 3 of culture. Maximum proliferation of T cells was detected around day 6—suggestive of a primary T cell stimulation. Other researchers have shown that the murine AMLR peak response occurred around day 7 in primary cultures but, upon restimulation of T cells with fresh DCs, T cell proliferation peaked on day 3 (Weksler and Kozak, 1977).

T cell proliferation was greater in FBS compared to AS (Buchele and Hopfl, 1997). Therefore, a subset of the T cells proliferating in FBS might be specific for foreign serum-derived antigens presented by DCs. In contrast, T cells cultured with DCs in AS are likely responding to autoantigens from self-proteins (Narendran et al., 2004). This is supported by the kinetics of CFSE dilution, which shows that a small percentage of DC-stimulated T cells are $CFSE^{dim}$ at early time points and, by day 6 of culture, have markedly diluted the CFSE. In contrast, Con A-stimulated samples have a similar percentage of $CFSE^{dim}$ T cells by day 6, but these cells are 1 log brighter in their CFSE fluorescence. These data suggest that DCs stimulate a distinct, potentially autoreactive T cell population that undergoes multiple rounds of proliferation, while Con A stimulates fewer replication cycles in a larger, non-specific population of cells. The presence of autoreactive T cells in the peripheral blood can be explained by the known imperfections in thymic negative selection and the suspected crossreactivity of the TCR (Mason. 1998; D'Orsogna et al., 2010). Also, DCs may activate autoreactive T cells by presenting cryptic epitopes resulting from caspase modification of self-proteins from nearby apoptotic cells (Amel Kashipaz et al., 2002; Chernysheva et al., 2002; Barat et al., 2009).

We became interested in the presence of Tregs in our equine DC/T cell co-cultures because Tregs are specific for self-antigen and because of the aforementioned suppressive capacity of AMLR-stimulated human T cells. We found that a subset of the DC-stimulated, proliferating T cells (5 to 15%) expressed the Treg transcription factor FoxP3. It was unclear

whether these cells were truly Tregs because in humans, unlike in mice, conventional T cells transiently upregulate FOXP3 upon cellular activation in the absence of exogenous TGF-β (Garden et al., 2011). Similarly, increased FoxP3 levels were reported in mitogen-stimulated canine and equine PBMCs, suggesting that FoxP3 gene expression is regulated similarly to humans in these species (Mizuno et al., 2009; Robbin et al., 2011). To further investigate the role of FoxP3 in the DC-stimulated samples, we measured cytokine production and found that approximately equivalent numbers of the proliferating FoxP3+ cells were capable of producing IFN-γ, IL-4, and IL-10. This finding confirms that suppressive and effector phenotypes exist concurrently within the FoxP3+ population (Robbin et al., 2011). However, compared to the proliferating FoxP3- cells, more of the proliferating FoxP3+ cells produced IL-10 and fewer produced IFN-γ. If FoxP3 were simply a marker of activation with no functional significance, the cytokine profile of both groups of cells would be identical. Instead, the FoxP3+ cells produced a more immunosuppressive cytokine profile, demonstrating that FoxP3 expression was associated with a regulatory transcriptional program in at least some of the proliferating cells. This is likely mediated by the inhibitory effect of FoxP3 on IFN-γ expression (Bettelli et al., 2005). Interestingly, a similar regulatory cytokine profile was also enriched in proliferating FoxP3+ cells following polyclonal stimulation with Con A. Therefore, the expansion of FoxP3+ cells and the associated alterations in cytokine production are not induced by DC-specific signals but are, instead, an inherent characteristic of equine T cell activation and proliferation. Measuring TGF-β production by these cells would also be interesting since production of this cytokine is a specific marker of regulatory activity, but this was not performed due to a lack of appropriate reagents.

The FoxP3+ cells following Con A or DC stimulation either represent expansion of pre-existing nTregs, induction of FoxP3 in activated conventional T cells, or a combination of the two. Although Con A stimulation induces some expansion of pre-existing nTreg populations in the dog, the vast majority of the FoxP3+ cells following polyclonal mitogenic stimulation are activated conventional T cells (Pinheiro et al., 2011). In addition, because nTregs do not proliferate readily, the $CFSE^{dim}$FoxP3+ cells analyzed in our experiments likely represent activated conventional T cells (Prochazkova et al., 2009). Future analysis of expression of Helios (a recently described nTreg-specific transcription factor) might verify this distinction (Thornton et al., 2010). Taken together, these results indicate that FoxP3 expression in activated equine conventional T cells is associated with a regulatory cytokine profile. The demonstration that FoxP3 is upregulated following DC stimulation proves that this effect is not mitogen-exclusive and supports the concept that FoxP3 might be induced during conventional T cell stimulation *in vivo*.

Conflicting results have been reported in experiments measuring the suppressive capabilities of human FOXP3+ conventional T cells (Walker et al., 2003; Pillai et al., 2007; Mahic et al., 2008; Kmieciak et al., 2009; Miyara and Sakaguchi, 2011). The functional immunosuppressive significance of transient, activation-induced FOXP3 expression is, therefore, suspect. However, the possibility that FOXP3+ effector T cells help to fine-tune the developing immune response is an attractive hypothesis that cannot be discounted—particularly because transduction of FOXP3 in naïve human T cells imparts a regulatory phenotype (Yagi et al., 2004; Mahic et al., 2008). This is supported by the recent finding that stimulation of conventional equine T cells induced a population of CD4+CD25+ T cells that were functionally suppressive (Hamza et al., 2011). However, these cells were activated in the presence of TGF-β, so they may

represent true Tregs rather than activated conventional T cells with transient Treg-like characteristics (Pillai et al., 2007). In other studies, activation-induced FOXP3 expression was insufficient to inhibit IFN-γ production by human effector T cells (IL-4 and IL-10 were not measured) (Gavin et al., 2006; Allan et al., 2007). This finding likely contrasts with our data because our analysis focused exclusively on the proliferating cells rather than both proliferating and non-proliferating populations. This allowed us to measure FoxP3 and cytokine expression only in the relevant, DC- or Con A-responsive cells.

We report herein that equine monocyte-derived DCs are capable of inducing the AMLR. This response should be considered when measuring the ability of DCs to induce an antigen-specific T cell response *in vitro*. We also found that equine DCs stimulate proliferation of autologous, potentially autoreactive helper T cells and that a subset of these cells exhibits activation-induced FoxP3 expression, which is associated with an immunoregulatory cytokine profile. Such activation-induced FoxP3 expression is an inherent component of proliferating equine T cells and might help regulate the developing immune response *in vivo*. These findings are relevant to the human immune system because FoxP3 expression is regulated similarly in humans and horses.

3.6. Acknowledgements

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CHAPTER 4:

EQUINE MONOCYTE-DERIVED DENDRITIC CELLS INDUCE KLH-SPECIFIC PROLIFERATION OF AUTOLOGOUS LYMPHOCYTES

4.1. Summary

Dendritic cells (DCs) serve a critical role in the induction and orchestration of the adaptive immune response. Therefore, the ability to analyze the interactions between equine DCs and T cells will greatly enhance our understanding of the equine immune system. We have developed a method of co-culturing relatively pure populations of equine monocyte-derived DCs with autologous, 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-stained T cells in the presence or absence of the experimental antigen keyhole limpet hemocyanin (KLH). Cellular proliferation, surface marker expression, and cytokine production were analyzed by multi-color flow cytometry. We have demonstrated for the first time that equine monocyte-derived DCs are capable of inducing antigen-specific proliferation of unprimed, autologous lymphocytes. However, this response could not be characterized further due to high levels of background proliferation in DC/T cell co-cultures without KLH. Attempts to reduce the background proliferation using serum-free conditions were not successful. Instead, horses were vaccinated with KLH to increase the DC-induced, KLH-specific response *in vitro*. The proliferating, KLH-specific cells consisted of both helper T cells and non-T cells, which were shown to be Syndecan-1-positive plasma cells. In addition, the KLH-responsive population was enriched for IL-4-competent cells and depleted of IFN-γ-competent cells. These results highlight the value of CFSE-based proliferation assays for functional characterization of equine monocyte-derived DCs. In addition, they demonstrate the potent antigen-presenting capabilities of equine DCs, which validate their potential use as a cellular vaccine adjuvant.

4.2. Introduction

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that serve as sentinels of the immune system by continuously sampling antigens in the peripheral tissues. Following maturation, DCs are uniquely capable of optimally sensitizing naïve antigen-specific T cells in the lymph node paracortex to generate an adaptive immune response. DC-derived signals promote the development of a variety of effector T cell responses, anergy, or tolerance (Mellman and Steinman, 2001). Therefore, a detailed analysis of the antigen-specific interactions between equine DCs and T cells will enhance our understanding of the immune response to infection or vaccination and may promote the development of improved vaccination strategies (Ueno et al., 2010).

We are interested in characterizing the functionality of equine monocyte-derived DCs by measuring their ability to stimulate an antigen-specific adaptive immune response *in vitro*. We anticipate that this analysis will broaden our understanding of the equine immune response and validate the use of DCs as a cellular vaccine adjuvant. Most previous attempts to measure antigen-specific cellular immune responses in horses have been limited to the culture of peripheral blood mononuclear cells (PBMCs) with or without antigen (Fitzpatrick et al., 1992; Frayne and Stokes, 1995; McKelvie et al., 1998; Davis et al., 2008). These assays rely on peripheral blood APCs such as monocytes to stimulate antigen-specific lymphocytes. The use of monocyte-derived DCs may increase the sensitivity of these assays, since DCs express higher levels of MHC and co-stimulatory molecules (Schuurhuis et al., 2006). Initial attempts to measure equine DC-induced lymphocyte responses have utilized heterogeneous cell populations and have quantified proliferation via tritiated thymidine uptake, which does not permit phenotypic characterization of the responding cells (Siedek et al., 1997; Hammond et al., 1999;

Siedek et al., 1999). Furthermore, tritiated thymidine assays only detect proliferation that occurs in the last 6-24 hours of culture, and the sensitivity of these assays is often reduced by high levels of background signal from non-proliferating cells (Schneider et al., 2002). In addition, tritiated thymidine has been shown to be less sensitive for detecting antigen-specific lymphocyte proliferation *in vitro* compared to 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-based assays (Mannering et al., 2003).

We co-cultured relatively pure populations of equine monocyte-derived DCs with autologous CFSE-stained peripheral blood T cells in the presence or absence of keyhole limpet hemocyanin (KLH). KLH was used as our experimental antigen because the use of this protein in immunological assays has been well established (Harris and Markl, 1999). Furthermore, the frequency of peripheral blood T cells specific for KLH in naïve humans has been shown to be relatively high (between 1/23,800 and 1/52,631) (Gebel et al., 1983). Therefore, we hypothesized that KLH would be useful for measuring the ability of equine monocyte-derived DCs to stimulate an antigen-specific response in naïve and primed autologous T cells. The magnitude and nature of the DC-induced, KLH-specific response was monitored using multi-color flow cytometry to measure cellular proliferation, surface marker expression, and cytokine production.

4.3. Materials and Methods

4.3.1. Monocyte isolation and generation of dendritic cells

Peripheral blood was collected into 10 mL heparinized Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) by jugular venipuncture from healthy adult horses according to an approved Institutional Animal Care and Use Committee (IACUC) protocol. Peripheral blood

mononuclear cells (PBMCs) were isolated via 1077 Ficoll-paque (Amershan Biosciences, Piscataway, NJ) density centrifugation at 700 x g for 15 min. Monocyte isolation was performed by CD14+ selection using an LS column (Miltenyi Biotech; Bergisch Gladback, Germany), as previously described (Chapter 3). Three million CD14+ cells were plated onto a 35 mm Petri dish (Fisher Scientific, Pittsburgh, PA) in 3 mLs of Aim-V® medium (Gibco-Invitrogen, Grand Island, NY) with 10% autologous serum (AS) and allowed to adhere for 1 hour at 5% CO_2 and 37°C. Nonadherent cells were removed by gently washing with DMEM-F12 media (Gibco-Invitrogen). The adherent monocyte-enriched population (approximately 2.5 x 10^6 cells) was cultured in 3 mLs of DMEM-F12 complete medium (Gibco-Invitrogen) with 10% AS (DCs), DMEM-F12 complete medium with 10% AS and 1 μg/mL lipopolysaccharide (Sigma-Aldrich, St. Louis, MO) (LPS DCs), or serum-free X-VIVO™ 15 Medium (Lonza(Byers et al., 2009), Walkersville, MD) (X-VIVO™ DCs). All three types of DC media were supplemented with 1X antibiotics/antimycotics (Gibco-Invitrogen), 10 ng/mL recombinant equine IL-4 (rEqIL-4) (kindly provided by Dr. David Horohov, University of Kentucky, Lexington, KY), and 50 ng/mL recombinant human granulocyte-macrophage colony-stimulating factor (rHuGM-CSF) (R&D Systems, Minneapolis, MN) for 3 days at 5% CO_2 and 37°C. For comparison, macrophages were generated by culturing monocytes in DMEM-F12 complete medium with 10% AS, 1X antibiotics/antimycotics, and no exogenous cytokines.

4.3.2. Autologous T cell isolation and CFSE staining

On the third day of DC culture, blood was collected from the same horse and PBMCs were isolated via 1077 Ficoll-paque density centrifugation. T cells were enriched by negative selection over an LD column (Miltenyi biotech), as previously described (Chapter 3). The

purified T cells were washed in PBS, resuspended in 0.25 μM 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich), and incubated on a spinning rack at room temperature in the dark for 10 min (Flaminio et al., 2004). An equal volume of fetal bovine serum (FBS) (Gibco-Invitrogen) was added to inactivate the extracellular CFSE, and the cells were washed 3 times in PBS then resuspended in lymphocyte proliferation medium consisting of RPMI 1640 medium (Gibco-Invitrogen) enriched with 10% AS, 25 μM 2-mercaptoethanol (Sigma-Aldrich), and 1X antibiotics/antimycotics (Gibco-Invitrogen).

4.3.3. APC harvest

On day 3 of culture, non-adherent DCs and macrophages were removed by washing the well with PBS and saved. Adherent cells were treated with 1 mL of Accumax™ (Millipore, Temecula, CA) at room temperature for 5 min and gently removed with a cell lifter. The adherent and non-adherent cells were pooled, washed in PBS, and resuspended in lymphocyte proliferation medium. Cell viability was assessed by 0.4% Trypan blue exclusion (Gibco-Invitrogen).

4.3.4. Cell culture

Purified, CFSE-stained T cells were plated in a 96-well, flat bottom tissue culture plate (Becton Dickinson) at a concentration of 3×10^5 cells in 300 μL of lymphocyte proliferation medium. 6×10^3 DCs, LPS DCs, or macrophages were added to the appropriate wells (1 APC:50 T cells). Positive control wells were treated with 5 μg/mL Concanavalin A (Con A) (Sigma-Aldrich). All treatments were performed with and without 25 μg/mL of KLH (Sigma-Aldrich), in triplicate for each horse. Samples assayed for cytokine production were treated with

40 ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich), 1 μg/mL ionomycin (Sigma-Aldrich), and 10 μg/mL brefeldin A (Sigma-Aldrich) for the last 5 hours of culture.

4.3.5. Flow cytometry

All samples were analyzed on a FACSCalibur® flow cytometer (Becton Dickinson) equipped with argon-ion and red-diode lasers. DCs, LPS DCs, and macrophages were harvested after 3 days of culture as described, and surface marker expression was measured by incubating with monoclonal antibodies (mAbs) against equine MHC class II (CZ11 hybridoma clone 130.8 E8D9, kindly provided by Dr. Douglas Antczak, Cornell University, Ithaca, NY), equine CD14 (hybridoma clone 105, kindly provided by Dr. Bettina Wagner, Cornell University, Ithaca, NY), human CD86 (PE-conjugated hybridoma clone 2331, Becton Dickinson), or human CD206 (PE-conjugated hybridoma clone 3.29B1.10, Beckman Coulter, Fullerton, CA). Cells were washed, and the unconjugated primary mAbs were labeled with PE-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch, West Grove, PA). T cells were harvested and proliferation was quantified by measuring the percent of cells in the lymphocyte gated area that were $CFSE^{dim}$. Cell surface marker expression was analyzed by incubating cells with mAbs against equine CD3 (hybridoma clone F6G3.3, Stott Lab, UC Davis), CD4 (hybridoma clone GB61A, VMRD, Pullman, WA), CD8 (hybridoma clone HT14A, VMRD), CD19 (hybridoma clone CZ2.1, kindly provided by Dr. Douglas Antczak), or CD172a (hybridoma clone SWC3, VMRD), followed by a PE-conjugated anti-mouse IgG secondary antibody (Jackson Immunoresearch). In one experiment, cells were labeled with polyclonal goat anti-human Syndecan-1 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, California), followed by a PE-conjugated donkey anti-goat IgG secondary antibody (Jackson Immunoresearch). Intracellular protein expression

was measured following permeabilization of cells with a saponin-based reagent (Cytofix/Cytoperm™ kit, Becton Dickinson). Cells were stained for expression of Alexa 647-conjugated anti-equine IL-4 (hybridoma clone 12H8, Wagner Lab, Cornell University), anti-equine IL-10 (hybridoma clone 492-2, Wagner Lab, Cornell University), or anti-bovine interferon-γ (hybridoma clone CC302, AbD Serotec, Oxford, UK).

4.3.6. Vaccination

KLH vaccines were prepared by diluting 2 mg of KLH (Sigma-Aldrich) in sterile PBS to a final volume of 1 mL. Three horses were each vaccinated intramuscularly in the neck 3 times at 2 week intervals. Serum samples were collected periodically and stored at -20◦C.

4.3.7. Anti-KLH antibody ELISA

A 96-well ELISA plate was coated overnight at 4◦C with 100 µL of 2.5 µg/mL KLH (Sigma) in 0.1M carbonate buffer. The plate was washed with 0.05% Tween 20 (Sigma) in TBS and blocked with 1% BSA in TBS (Sigma). Serum samples were diluted 1:500 with TBS containing 1% BSA and 0.05% Tween 20 and incubated in the KLH-coated wells for 1 hour at room temperature. A goat anti-horse IgG (heavy and light chain)-peroxidase detection antibody was used (Jackson Immunoresearch). The wells were read using a spectrophotometer at 450 nm, and data analyzed using Ascent Software (Thermo Electron Corporation, Vantaa, Finland). All samples were measured in duplicate.

4.3.8. Statistical analysis

Paired t tests were used to compare proliferation in the presence or absence of KLH and DC viability in different kinds of media. An overall p-value of 0.05 (2-sided) was used to indicate significance.

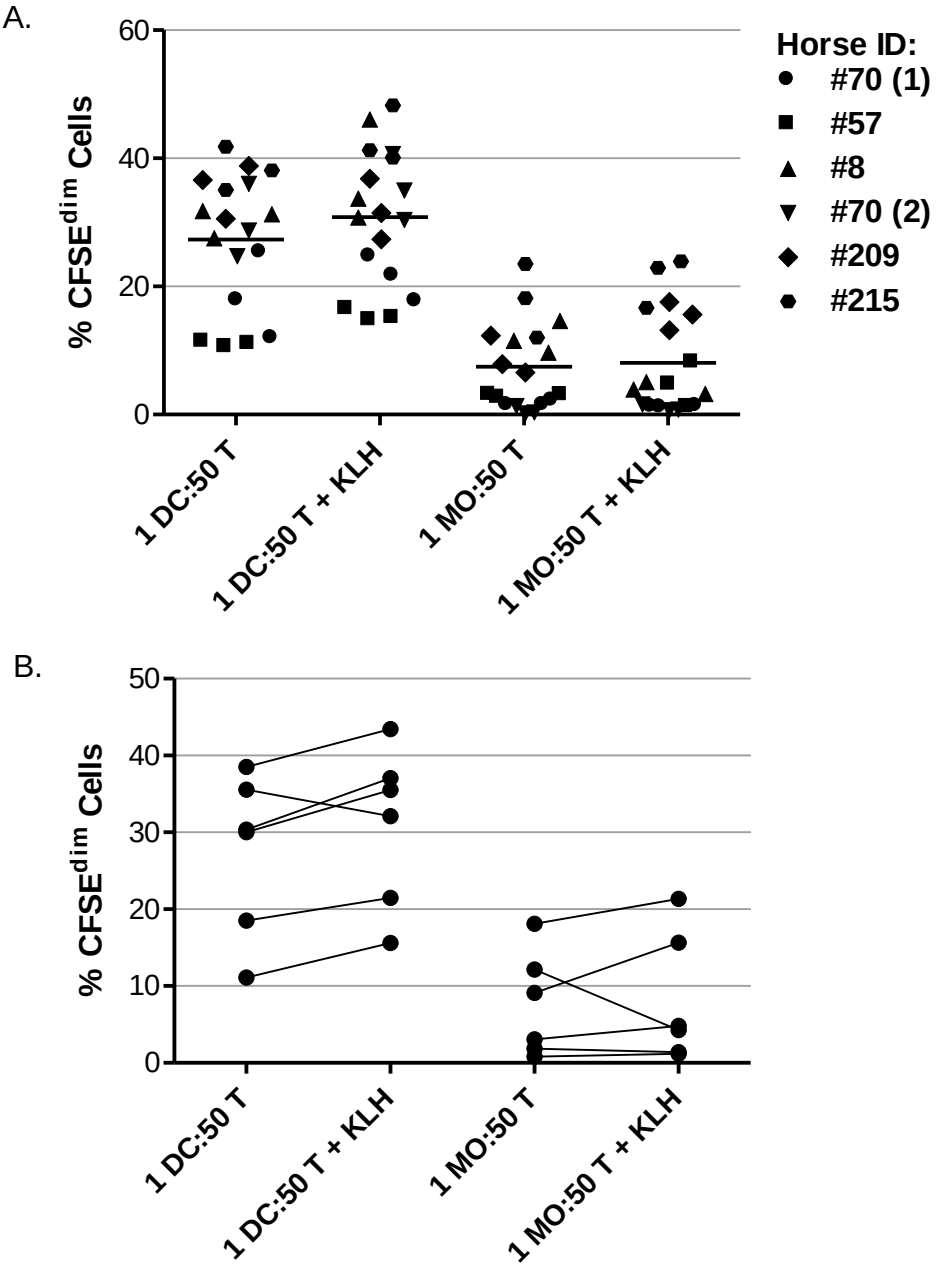
4.4. Results

4.4.1. Equine monocyte-derived DCs, but not macrophages, induce KLH-specific proliferation in naïve, autologous T cells

We attempted to further characterize equine monocyte-derived DCs by measuring their ability to stimulate naïve T cells in an antigen-specific manner. A small but fairly consistent increase in the percentage of proliferating cells was detected in DC/T cell co-cultures with KLH (Figure 4.1A). Mean values of triplicate samples for each horse were compared and found to be greater in co-cultures with KLH compared to those without KLH ($p < 0.05$) (Figure 4.1B). However, because the KLH-specific increase in proliferation was minor and did not withstand Bonferroni adjustment, it was of questionable biological relevance or experimental utility. In contrast, no increase in T cell proliferation was noted in macrophage/T cell co-cultures in the presence of KLH ($p > 0.05$).

Figure 4.1. DCs, but not macrophages, induce KLH-specific proliferation when cultured with autologous naïve T cells. CFSE-stained T cells from 5 horses (1 horse was used twice) were cultured with autologous DCs or macrophages in medium containing autologous serum with or without 25 μg/mL KLH. Cells were harvested on day 6 or 7 of culture, and the percentage of cells within the lymphocyte gate that were proliferating ($CFSE^{dim}$) was measured by flow cytometry. (A) Triplicate values from each horse are shown. Bars represent mean values for each group. (B) Each data point represents the mean of triplicate samples. Lines indicate that values from cultures with and without KLH are from the same horse.

Figure 4.1. DCs, but not macrophages, induce KLH-specific proliferation when cultured with autologous naïve T cells (continued).

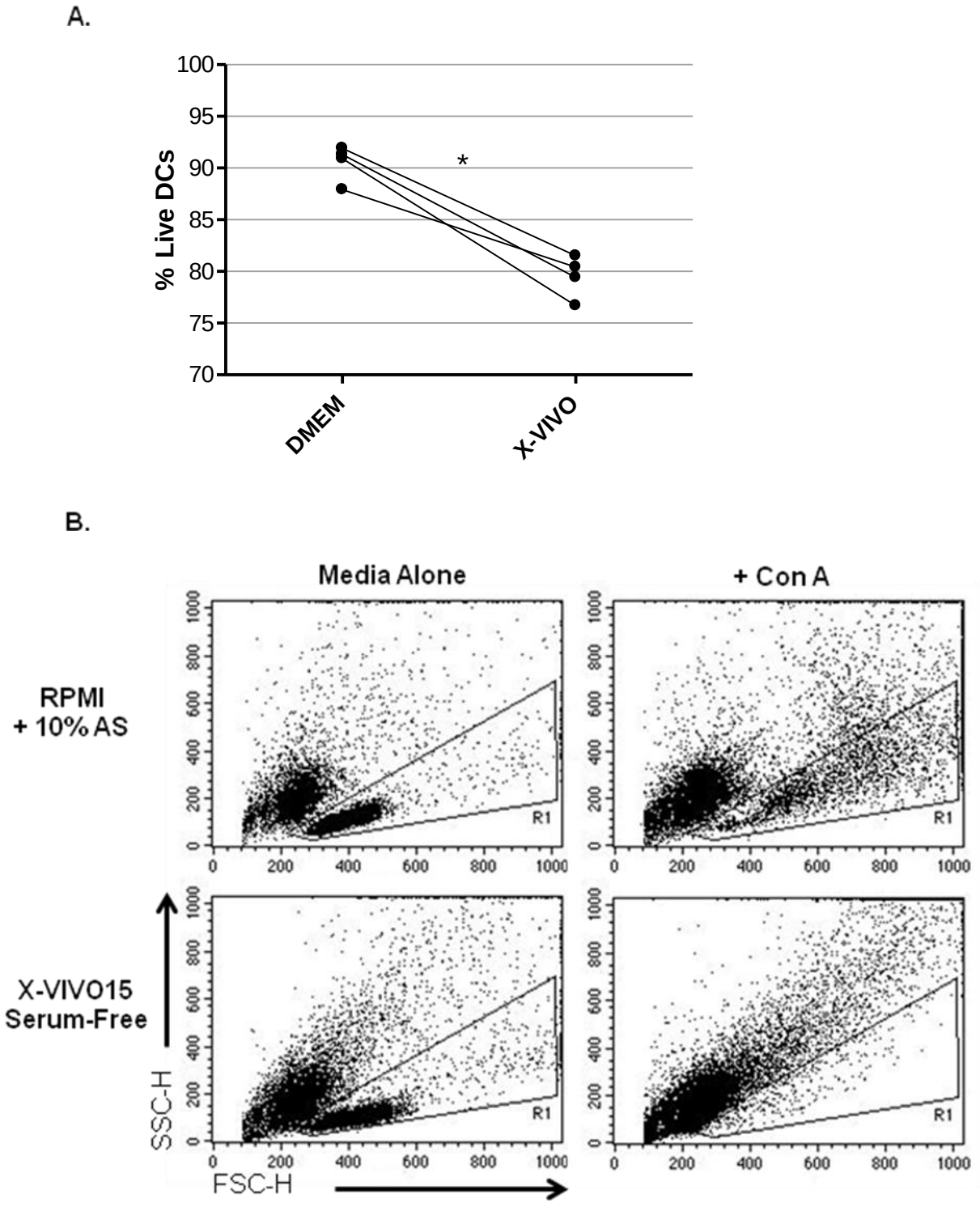


4.4.2. Serum-free medium does not support equine cell culture

High levels of "background" proliferation were detected in the DC/T cell co-cultures without KLH (Figure 4.1 and Chapter 3). We were concerned that this background proliferation was preventing us from measuring KLH-specific T cell proliferation. In similar experiments with human cells, researchers have used serum-free medium to reduce T cell stimulation in control samples (Byers et al., 2009). We assessed the ability of serum-free X-VIVO™ 15 medium to support the culture of equine DCs and T cells. We anticipated that co-culturing these cells in a serum-free environment would reduce non-KLH-specific proliferation. Viability of DCs was reduced significantly (mean 12.2% decrease) when cultured for 3-5 days in serum-free X-VIVO™ medium compared to DMEM F12 medium with 10% autologous serum (AS), as determined by 0.4% Trypan blue exclusion ($p < 0.005$) (Figure 4.2A). PBMCs from a single horse were cultured for 4 days in either RPMI medium with 10% AS or serum-free X-VIVO™ medium and analyzed by flow cytometry. Comparable numbers of non-stimulated PBMCs were present in the live lymphocyte gate regardless of culture media used (Figure 4.2B). However, the majority of the cells in the lymphocyte gate shifted into the non-viable cell region after stimulation with Con A in the serum-free medium but not in RPMI with 10% AS. Trypan blue exclusion revealed that 73.7% of the Con A-stimulated PBMCs in RPMI with 10% AS were alive compared to just 26.4% of cells in the serum-free medium.

Figure 4.2. Equine DCs and stimulated T cells have decreased viability in serum-free medium. (A) DCs from 4 different horses were harvested after 3-5 days of culture in DMEM F12 medium containing 10% autologous serum or serum-free X-VIVO™ 15 medium and assessed for cellular viability by 0.4% Trypan blue exclusion. Lines indicate DCs are from the same horse. (B) PBMCs from a single horse were cultured in 250 μL RPMI medium containing 10% autologous serum or serum-free X-VIVO™ 15 medium with or without Concanavalin A stimulation (2 x 10^5 cells/well). Cells were harvested after 4 days of culture and analyzed by flow cytometry. R1 indicates the live lymphocyte region. * $p < 0.005$.

Figure 4.2. Equine DCs and stimulated T cells have decreased viability in serum-free medium (continued).



4.4.3. Vaccination of horses with KLH induces anti-KLH IgG antibody production

We were unable to reduce the non-KLH-specific T cell proliferation in our co-cultures because the required serum-free conditions could not adequately support equine cell culture. Therefore, we hypothesized that the KLH-specific response *in vitro* could be amplified by vaccinating the horses with KLH to increase the frequency of KLH-specific T cells in the DC/T cell co-culture system. We anticipated that increasing the KLH-specific proliferation above background in this manner would facilitate further characterization of the DC-induced, antigen-specific adaptive immune response. To this end, 3 horses were vaccinated intramuscularly (IM) with 2 mg of non-adjuvanted KLH protein. Each horse was vaccinated 3 times at 2 week intervals, and serum samples were taken periodically to monitor KLH-specific antibody production. All 3 horses produced detectable levels of anti-KLH immunoglobulins following the vaccination series (Figure 4.3). The KLH titer was greatest in horse #70.

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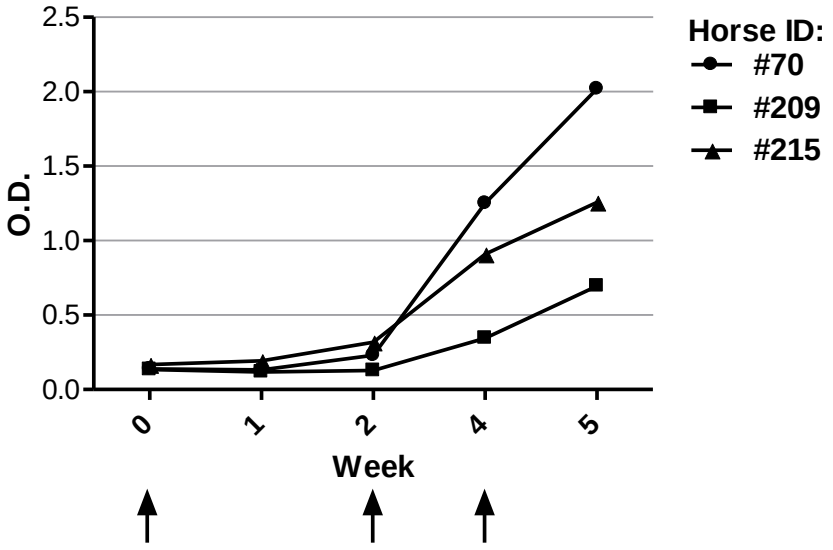


Figure 4.3. Vaccination of horses with KLH induces anti-KLH antibody production. Three horses were each vaccinated intramuscularly with 2 mg of purified KLH antigen on weeks 0, 2, and 4 (arrows). Blood was collected from each horse on weeks 0, 1, 2, 4, and 5, and serum anti-KLH antibody levels were measured by ELISA. Values indicate the mean of duplicate samples. Results are reported in optical density (O.D.)

4.4.4. DCs and macrophages induce KLH-specific proliferation of primed T cells

We used primed T cells from the KLH-vaccinated horses to compare the ability of DCs and macrophages to induce a KLH-specific response. We also examined the effect of stimulating DCs with LPS on their ability to present antigen. Ten days after the final KLH vaccination, T cells were isolated, stained with CFSE, and co-cultured with autologous macrophages, DCs, or LPS-stimulated DCs in medium with or without 25 μg/mL of KLH (Figure 4.4A). Samples were performed in triplicate and harvested on days 4 and 6 for flow cytometric analysis. T cell proliferation, surface phenotype, and cytokine production were analyzed to characterize the magnitude and nature of the KLH-specific T cell response generated by the three different APC groups.

On day 4 of culture, KLH-specific proliferation was detected in cells cultured with non-stimulated DCs and LPS-stimulated DCs (Figure 4.4B). The magnitude of this response varied by horse and was greatest in horse #70. In contrast, minimal KLH-specific proliferation was detected in cells cultured with macrophages, and none was detected in T cells cultured with KLH in the absence of APCs. On day 6 of culture, fairly high levels of proliferation were detected in T cells cultured with DCs or LPS-stimulated DCs in the absence of KLH (Figure 4.4C). However, proliferation increased over these background levels when the cells were cultured with non-stimulated DCs or LPS-stimulated DCs in the presence of KLH. KLH-specific proliferation was also detected in cells cultured with macrophages. The KLH-specific proliferation was markedly increased compared to that seen with naïve T cells stimulated with either DCs or macrophages (Figure 4.1A). The relative KLH-specific increase in proliferation was again greatest in horse #70, which was partially due to lower levels of background proliferation in T cells from this horse. A small increase in proliferation was detected in T cells cultured with

KLH in the absence of added macrophages or DCs. Treatment of DCs with LPS did not influence their ability to induce KLH-specific or background proliferation on day 4 or day 6. This is consistent with the flow cytometric phenotype of these cells, which revealed no differences in surface marker expression between non-stimulated and LPS-stimulated DCs (Figure 4.5).

Figure 4.4. Ability of APCs to induce KLH-specific proliferation of primed T cells. (A) T cells were isolated from 3 horses 10 days after the final KLH vaccination, stained with CFSE, and cultured alone or with macrophages, non-stimulated DCs, or LPS-stimulated DCs in medium containing autologous serum with or without 25 μg/mL of KLH. All samples were performed in triplicate. Cells were harvested on day 4 (B) or 6 (C) of culture, and the percent of cells within the lymphocyte gate that were proliferating ($CFSE^{dim}$) was measured by flow cytometry. Bars represent mean values. Data from T cell only culture samples with and without KLH from horse #215 on day 4 are missing.

Figure 4.4. Ability of APCs to induce KLH-specific proliferation of primed T cells (continued).

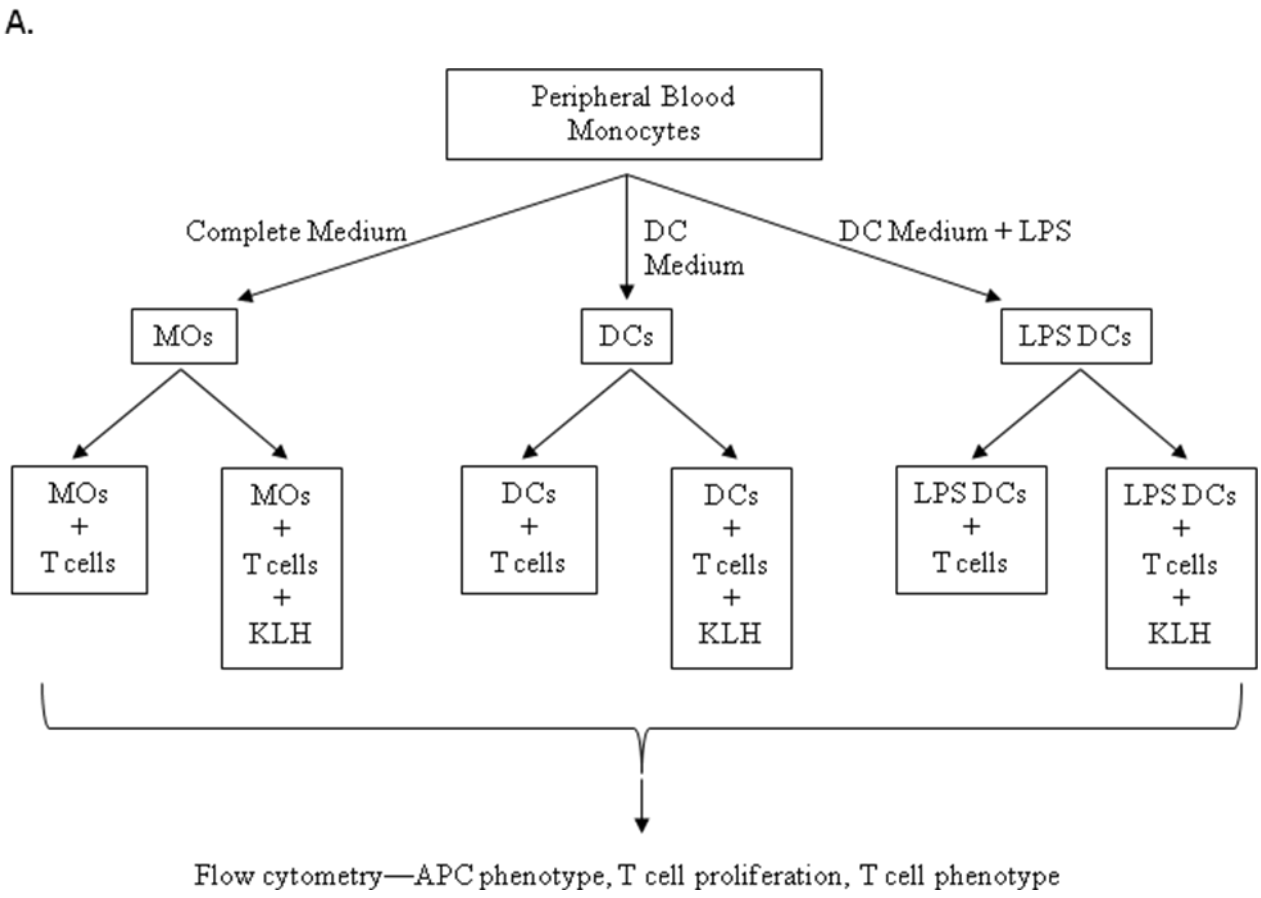
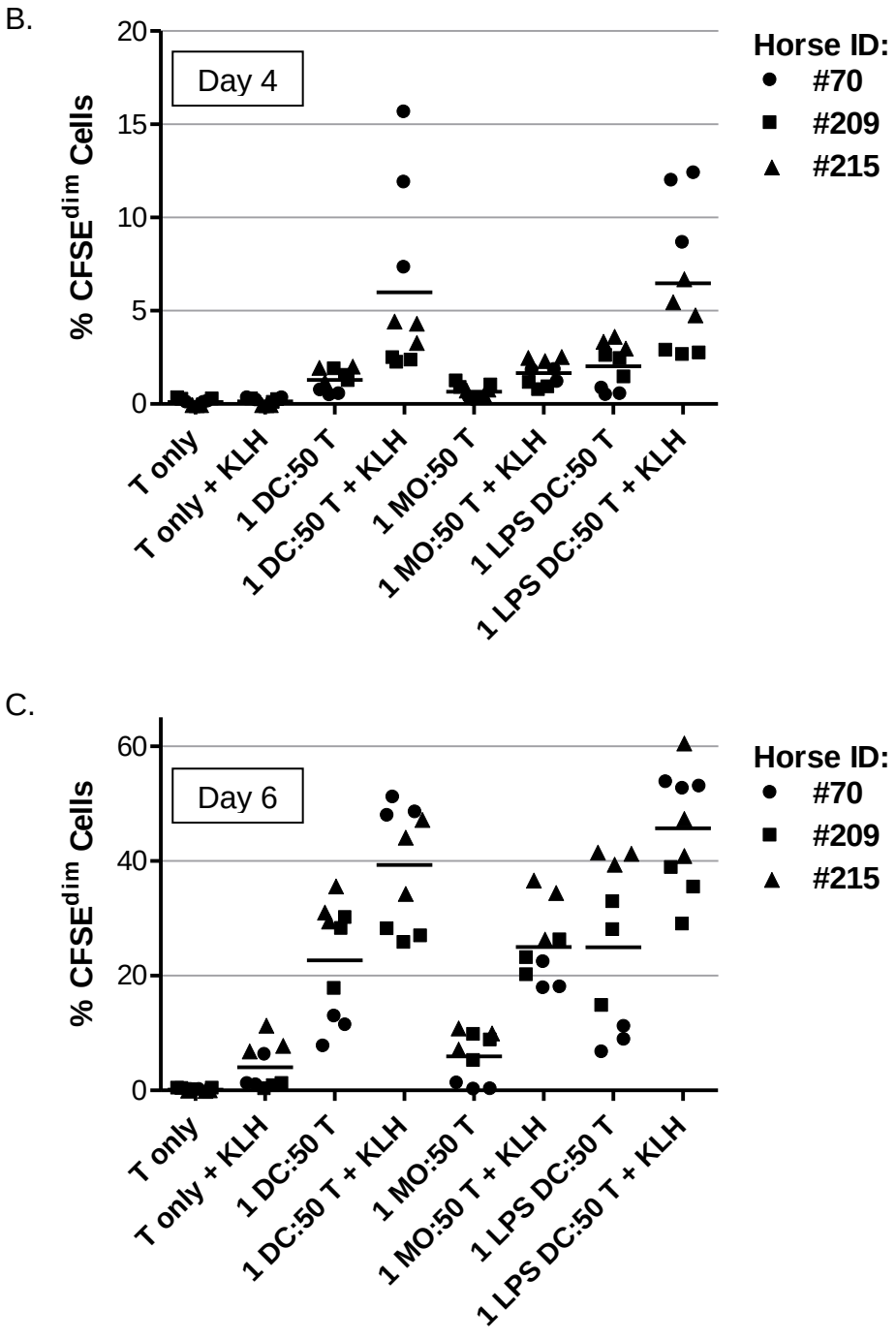


Figure 4.4. Ability of APCs to induce KLH-specific proliferation of primed T cells (continued).



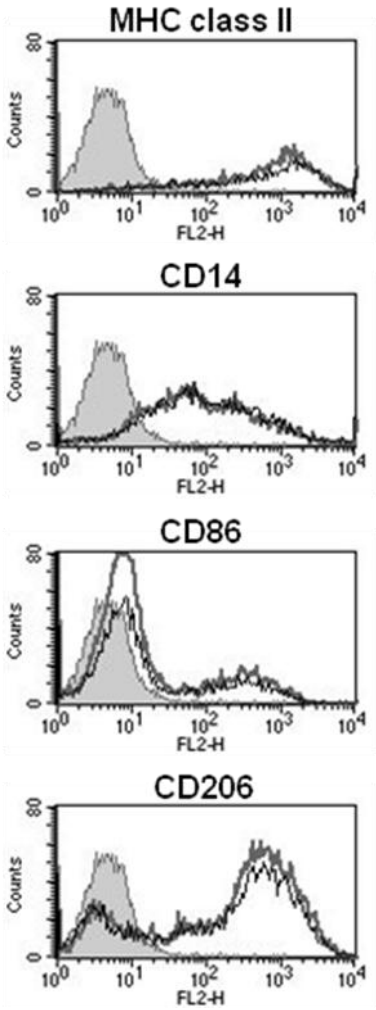


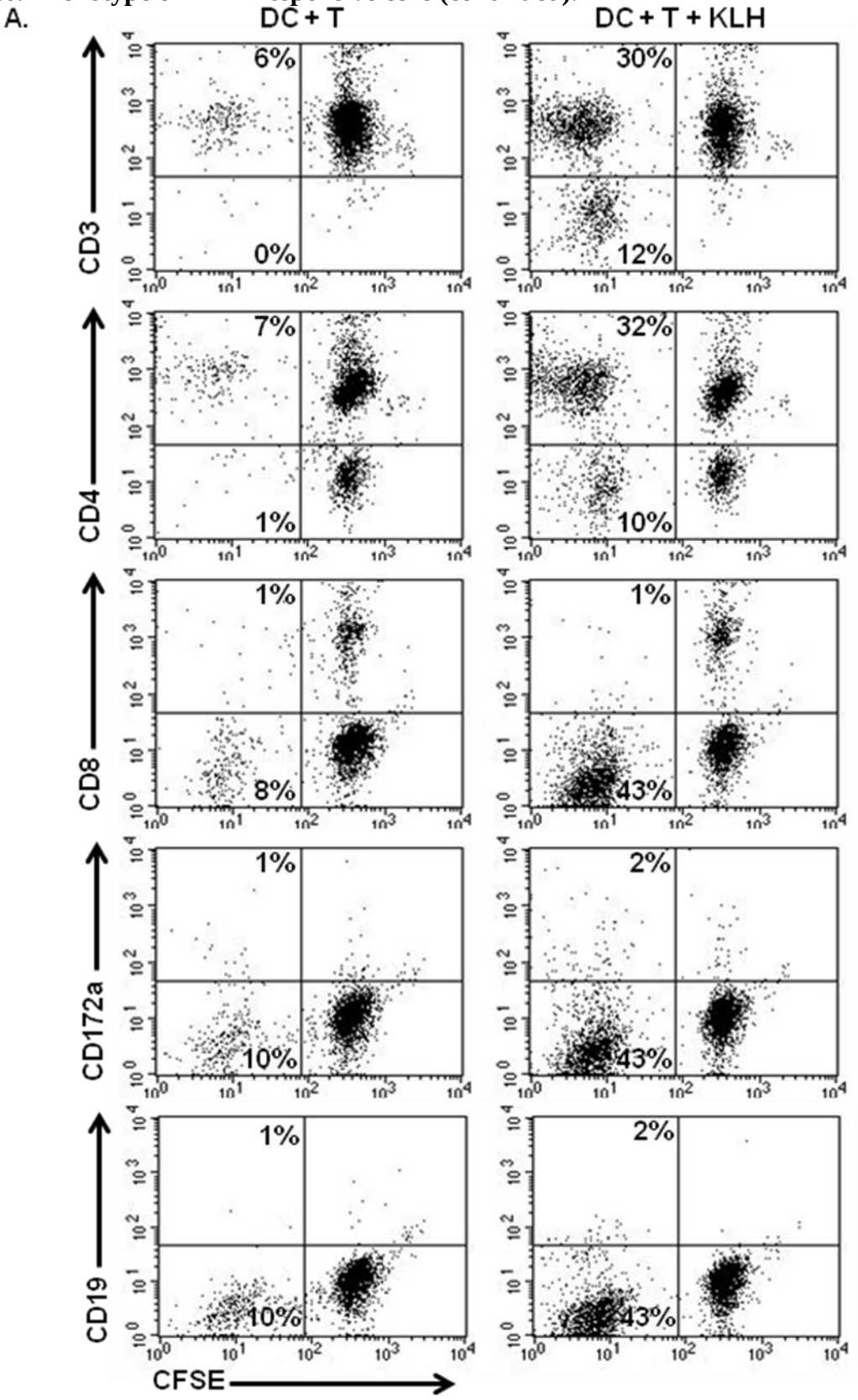
Figure 4.5. Phenotype of non-stimulated and LPS-stimulated DCs. Peripheral blood monocytes were cultured in DC medium for 3 days. Cells were either non-stimulated (DCs, thin black line) or treated with 1 μg/mL LPS for the last 24 hours of culture (LPS DCs, wide gray line). Cells were harvested and surface marker expression was analyzed by flow cytometry. Shaded regions indicate the isotype control. Data are representative of 3 independent experiments.

4.4.5. KLH-specific proliferation occurs in T cells and non-T cells co-cultured with DCs

T cells from the DC/T cell co-cultures with or without KLH were labeled with cell surface markers, and the phenotype of the proliferating, KLH-specific cells was analyzed. As noted previously (Chapter 3), the T cells proliferating in response to DCs in the absence of KLH were primarily CD3+, CD4+, CD8-, CD172a-, CD19- T cells (Figure 4.6). Unexpectedly, the T cells proliferating in DC/T cell co-cultures in the presence of KLH also contained a population of CD3-, CD4-, CD8-, CD172a-, CD19- cells of uncertain identity. This non-T cell population was detected in all 3 horses. Therefore, when DCs were cultured with T cells from KLH-vaccinated horses in the absence of KLH, all proliferating cells were T cells. In contrast, when DCs were cultured with T cells from KLH-vaccinated horses in the presence of KLH, the proliferation population consisted of both T cells and non-T cells. Smaller numbers of these cells were also present in co-cultures with macrophages (data not shown).

Figure 4.6. Surface marker expression of KLH-responsive cells. CFSE-stained T cells from KLH-vaccinated horses were cultured with DCs in medium with or without 25 μg/mL KLH. Cells were harvested after 6 days of culture, stained for surface expression of various lineage-specific markers, and analyzed by flow cytometry. All analyses were restricted to cells within the lymphocyte gate. Data shown are from horse #70. Numbers indicate the percent of cells in the relevant quadrant.

Figure 4.6. Phenotype of KLH-responsive cells (continued).



4.4.6. Many of the KLH-responsive cells produce IL-4 when stimulated with PMA and ionomycin

We originally intended to measure cytokine production by KLH-specific, DC-stimulated T cells to assess helper T cell differentiation. However, this was complicated by the presence of proliferating, non-T cells in the co-cultures with KLH. Despite this complication, we decided to measure cytokine production to further define the KLH-responsive cells. To do so, we re-stimulated the co-cultures with PMA plus ionomycin and compared cytokine production by the $CFSE^{dim}$ population in the presence or absence of KLH (Figure 4.7). We found that more of the cells proliferating in co-cultures with KLH produced IL-4 and fewer produced IFN-γ. No cytokine production was detected in more than 50% of the cultured cells.

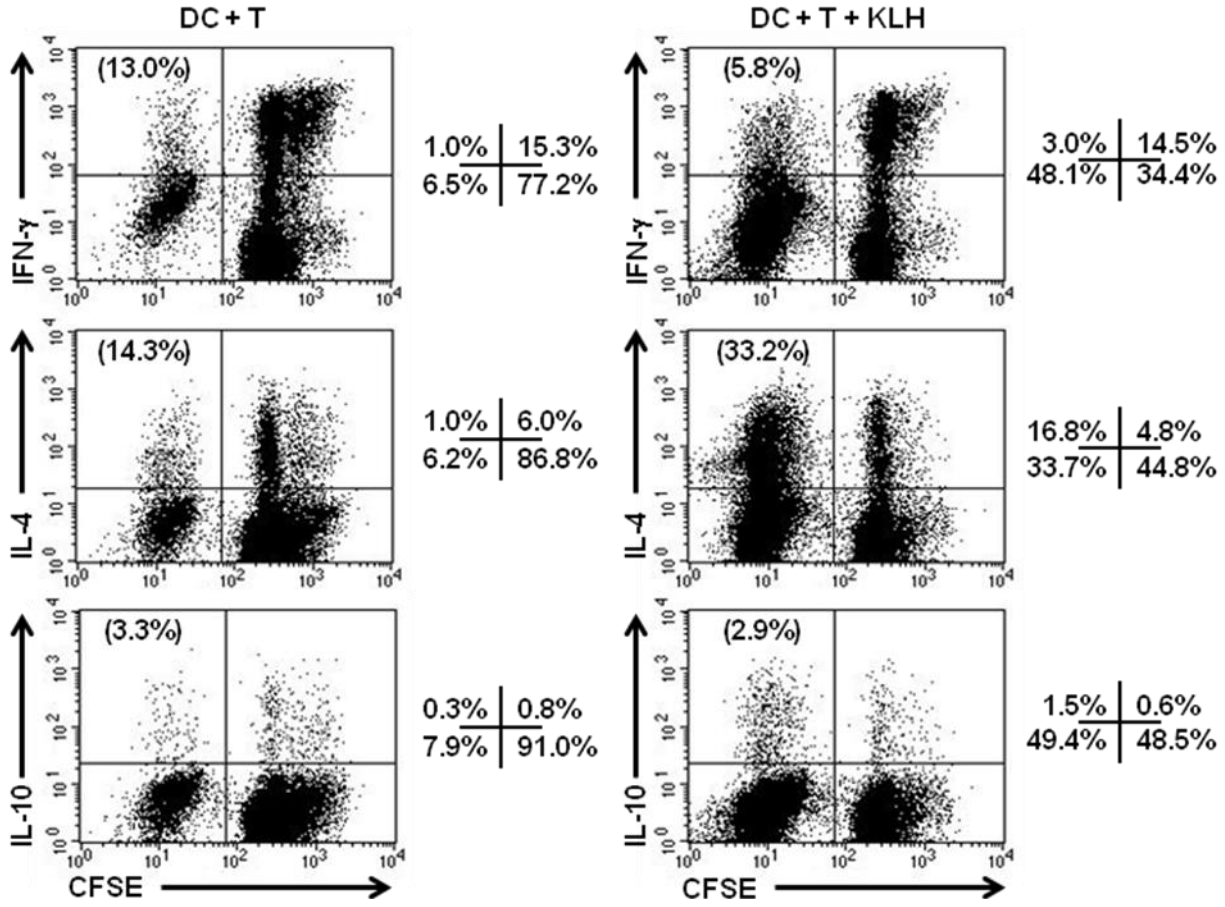


Figure 4.7. Cytokine production by KLH-responsive cells. CFSE-stained T cells from KLH-vaccinated horses were cultured with DCs in medium with or without 25 μg/mL KLH. Cells were harvested after 6 days of culture and treated with PMA, ionomycin and brefeldin A for the last 5 hours of culture. Cells were harvested, permeabilized, stained for cytokine production (IFN-γ, IL-4, or IL-10), and analyzed by flow cytometry. All analyses were restricted to cells within the lymphocyte gate. Values to the right of each graph indicate the percent of cells in each quadrant. Values in parenthesis indicate the percent of proliferating cells that are positive for the indicated cytokine. Data from horse #70 are shown because this horse had the strongest response to KLH. However, the data trends were the same for all 3 horses tested.

4.4.7. The proliferating, KLH-specific non-T cells express the plasma cell marker Syndecan-1

Horse #70 was re-vaccinated with 2 mg of KLH administered intramuscularly 132 days after the initial KLH vaccination (106 days after the previous KLH booster). T cells were isolated 10 days after vaccination and co-cultured with DCs in the presence or absence of 25 ug/mL KLH. Cell phenotype was determined by flow cytometry after 6 days of culture. Once again, only CD3+ T cells proliferated in response to DCs in the absence of KLH, and a population of both CD3+ T cells and CD3- non-T cells proliferated in co-cultures with KLH (Figure 4.8). Interestingly, the non-T cells were positive for the plasma cell marker, Syndecan-1.

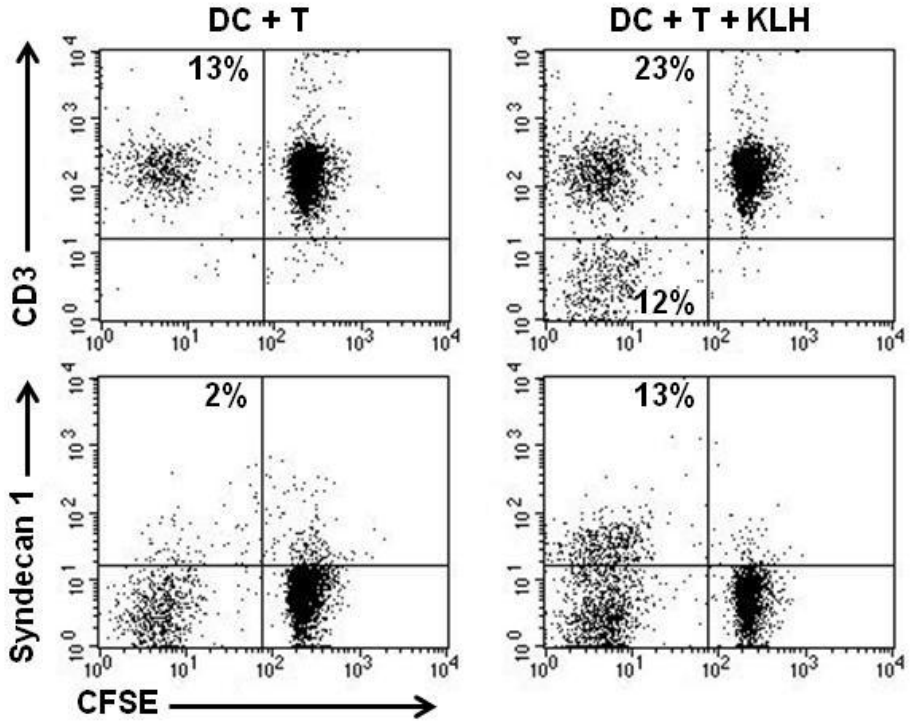


Figure 4.8. KLH-responsive, non-T cells are plasma cells. CFSE-stained T cells from KLH-vaccinated horse #70 were cultured with DCs in medium with or without 25 μg/mL KLH. Cells were harvested after 6 days of culture, stained for surface expression of CD3 or Syndecan-1, and analyzed by flow cytometry. All analyses were restricted to cells within the lymphocyte gate. Numbers indicate the percent of cells in the relevant quadrant.

4.5. Discussion

We have demonstrated for the first time that equine monocyte-derived DCs, but not macrophages, are capable of inducing antigen-specific proliferation of unprimed, autologous lymphocytes. This contributes to our understanding of equine DC function and supports their potential value as an immunological tool and cellular vaccine adjuvant. However, the detectable magnitude of this response was minimal due to a high level of background proliferation in co-cultures without KLH. We have previously characterized this response (Chapter 3) and speculate that it represents activation of auto-reactive, CD4+ T cells responding to self-antigens presented by DCs. Reducing this proliferation is necessary to prevent a masking of the primary lymphocyte response and permit a more detailed analysis of the responding cell population.

Other researchers have utilized serum-free conditions to reduce background proliferation in human DC/T cell co-cultures when measuring *in vitro* priming of naïve T cells (Moser et al., 2010). Cell viability stains and antibodies against markers specific for antigen-activated T cells, such as CD137 (4-1BB) or CD154 (CD40L), were also used to exclude irrelevant cell populations from these analyses (Frentsch et al., 2005; Wehler et al., 2008). We found that serum-free X-VIVO™ 15 medium was not sufficient to support the culture of equine leukocytes, particularly mitogen-activated lymphocytes. This likely occurred because X-VIVO™ 15 medium contains human proteins (including albumin, insulin, and transferrin) and is optimized for the culture of human cells. In addition, we were unable to restrict our analysis to include only live, antigen-activated T cells due to a lack of appropriate reagents, including antibodies to detect CD40L or CD25 (Robbin et al., 2011). The future development of equine-specific monoclonal antibodies and culture medium is necessary to improve the gating strategies and cell culture conditions that are required to enhance the sensitivity of this assay.

In lieu of such reagents, we vaccinated 3 horses with KLH to increase the frequency of KLH-specific lymphocytes in the peripheral blood. The magnitude of the KLH-specific proliferative response in DC/T cell co-cultures following KLH vaccination varied by horse, as has been seen in similar experiments using human cells (Carotenuto et al., 2009). Due to this high degree of variation, meaningful statistical analyses will require larger sample sizes. However, we successfully demonstrated that the use of primed T cells from vaccinated horses increased the detectable KLH-specific response induced by equine monocyte-derived DCs. Interestingly, the amount of KLH-specific proliferation detected *in vitro* from each horse was proportional to the serum antibody titer (Horse #70>#215>#209). This finding has also been reported in PBMC proliferation assays after vaccination of humans against hepatitis B (Leroux-Roels et al., 1994).

Macrophages were also capable of stimulating a KLH-specific response in our experiments, which contrasts with the data from unvaccinated horses and is consistent with a lower activation threshold for effector and memory lymphocytes (Croft et al., 1994; Croft. 1994). As demonstrated previously (Chapter 3), macrophages induced less background proliferation compared to DCs. Therefore, macrophages may be more appropriate than DCs for measuring secondary immune responses *in vitro,* as the relative increase in antigen-specific proliferation is greater with these cells (Zielinski et al., 2011). Alternatively, DC/T cell co-cultures should be analyzed at earlier time points or using a smaller DC:T cell ratio.

LPS treatment did not induce phenotypic maturation or enhance antigen-presenting capabilities of DCs, which supports the concept that multiple stimuli are required to induce full DC maturation (Mauel et al., 2006; Dietze et al., 2008). However, the DC/T cell co-culture assay presented in this report may be useful as an *in vitro* method to test the effect of such

stimuli and assess their potential use as vaccine adjuvants. For example, the effect of adding various adjuvants or combinations of adjuvants on the ability of DCs to induce antigen-specific T cell proliferation and differentiation could be determined.

Multi-color flow cytometric analysis revealed an increase in the percent of proliferating CD3+ T cells in co-cultures with KLH. All of the proliferating CD3+ cells were CD4+ helper T cells, indicating that cross-presentation of soluble KLH to CD8+ cells did not occur. However, a population of CD3- cells also proliferated when stimulated by DCs or MOs in the presence of KLH. This finding was unexpected because the original T cell population was enriched by negative selection of CD172a+ and CD21+ cells. The proliferating non-T cells in our co-cultures with KLH did not express specific markers for B cells (CD19) or monocytes/neutrophils (CD172a), but did express a plasma cell marker (Syndecan-1). Therefore, the proliferating cells in the DC/T cell co-cultures consist of both KLH-specific T cells and plasma cells. This is consistent with previous reports that B cell responses can be detected when culturing soluble antigens with human PBMCs (Ichikawa et al., 1999; Schneider et al., 2002).

Because plasma cells are terminally differentiated, nondividing cells, they are incapable of diluting CFSE if isolated directly from peripheral blood (Slifka et al., 1998). Therefore, we hypothesize that KLH-specific effector and/or memory B cells from the peripheral blood of vaccinated horses underwent expansion and differentiation to plasma cells when cultured with DCs and KLH (Bernasconi et al., 2002). This is supported by the presence of a small number of CD3- cells that were consistently detected within the lymphocyte gate after purification of peripheral blood T cells by negative selection (Figure 3.1C). It is interesting to note that minimal proliferation was detected in cultures of T cells with KLH in the absence of DCs or macrophages. Therefore, DCs and macrophages must provide signals, such as cytokines or

(more likely) activated KLH-specific T cells, that are needed for efficient proliferation of KLH-specific B cells. Initial attempts to measure an increase in KLH-specific antibodies from culture supernatants were unsuccessful because the antibodies were bound up by soluble KLH (data not shown). Using ELISPOT assays to detect anti-KLH antibody-secreting cells may permit further characterization of this cell population.

The proliferating cells in co-cultures with DCs and KLH were enriched for IL-4-competent cells and depleted of IFN-γ-competent cells. It is not possible to definitively identify the IL-4-producing cells because, as discussed, the proliferating cell population represents a mixture of helper T cells and plasma. Therefore, the KLH-specific, IL-4-producing cells may represent Th2 cells, plasma cells, or a combination of the two. Simultaneous measurement of CFSE, CD3, and IL-4 will be required to distinguish between these possibilities.

We have shown that equine monocyte-derived DCs are capable of inducing KLH-specific proliferation by naïve and primed autologous lymphocytes. Multi-color flow cytometric analysis revealed that many of the responding cells produced IL-4 and were composed of both CD4+ helper T cells and plasma cells. These experiments highlight the value of using CFSE-based proliferation assays for functional characterization of equine DCs. Furthermore, these results demonstrate the potent antigen-presenting capabilities of equine monocyte-derived DCs and support their potential use as cellular vaccine adjuvants to promote novel immunotherapeutic strategies.

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CHAPTER 5:

SENTINEL LYMPH NODE IDENTIFICATION IN THE HORSE

5.1. Summary

Vaccine antigen deposited in the periphery must be transported through local lymphatic vessels to the sentinel lymph node (SNL) for an effective immune response to develop. We have adapted a lymphoscintigraphic method commonly employed in human and small animal oncology patients to identify the vaccine-draining node in the horse. This technique will promote the study of the equine immune response to vaccination and guide the development of improved vaccination strategies that enhance delivery of antigen to the lymph node.

5.2. Introduction

Soluble vaccine antigen is deposited in the peripheral tissue, drains through the local lymphatics to the sentinel lymph node (SLN), and is taken up by lymph node-resident dendritic cells (DCs) (Itano et al., 2003). In addition, migratory DCs travel to the SLN after acquiring vaccine antigen near the site of injection (Romani et al., 2001). Consequently, antigen presentation to T cells occurs in the lymph node that drains the site of vaccination. If antigen remains in the periphery and does not reach secondary lymphoid organs, no immune response will develop (Zinkernagel et al., 1997). Therefore, the lymphatic system serves an essential immunologic function by transporting antigens, chemokines, and leukocytes throughout the body. Understanding the local lymphatic anatomy is important when designing effective vaccines or studying the immune response to vaccination (Swartz et al., 2008). In particular, SLN identification permits the analysis of vaccine antigen presentation *in situ* (Meijer et al., 2001).

SLN identification is commonly performed in human oncology patients with a variety of neoplasms, including mammary carcinoma and cutaneous melanoma (Leong et al., 2011). Once identified, the tumor-draining lymph node is biopsied and examined histopathologically for evidence of metastasis to guide tumor staging and treatment strategy. SLN identification has recently been reported to also improve clinical assessment of small animal oncology patients in veterinary medicine (Tuohy et al., 2009).

Reported techniques for SLN identification include the use of vital dyes (Morton et al., 1992), radiotracers (Alex and Krag, 1993), and microspheres (Ueno et al., 2005), and may utilize imaging techniques such as radiography, ultrasonography, magnetic resonance, and computed tomography (Clement and Luciani, 2004). Scintigraphy is a safe, non-invasive procedure that is

commonly performed in horses to diagnose a variety of skeletal and non-skeletal conditions (Archer et al., 2007a; Archer et al., 2007b). Therefore, we performed lymphoscintigraphy to identify a vaccine-draining lymph node in the horse. This technique will promote analysis of the immune response to vaccination and the development of improved vaccination strategies.

5.3. Materials and Methods

5.3.1. Animals

A healthy thoroughbred mare was selected for this experiment based on favorable disposition. All procedures were performed according to an approved Institutional Animal Care and Use Committee (IACUC) protocol.

5.3.2. Lymphoscintigraphy

Initial experiments to identify lymph nodes draining an intramuscular (i.m.) injection failed to detect a sentinel node. The technetium 99m (^{99m}Tc) sulfur colloid radiotracer was confined to the injection site, even at 5 hours post injection. In another experiment, we gave an i.m. injection of ^{99m}Tc bound to a dextran carrier and also failed to detect a sentinel node. The dextran carrier diffused rapidly throughout the tissues, possibly entering blood capillaries, and no lymphatic structures were visualized. Therefore, we repeated the experiment using the ^{99m}Tc sulfur colloid radiotracer delivered via subcutaneous (s.c.) injection.

A calculated dose of 0.55 mCi of filtered ($\leq$ 220 nm) ^{99m}Tc sulfur colloid (ParmaLogic, Sayre, PA) was injected s.c. in the caudodorsal region of the left side of the neck in a 0.4 mL injection volume; this is a commonly used site for vaccination in horses. The horse was sedated lightly with detomidine hydrochloride and imaged using a Technicare 438 (Technicare, Inc.,

Solon, OH) gamma camera. Images were analyzed with the NuQuest nuclear medicine computer (MEDX, Inc., Arlington Heights, IL). Lateral images were obtained at 20, 30, 40, and 70 min post injection. Ventral images were obtained at 45, 50, and 60 min post injection. Approximately 250,000 events were obtained per image. For anatomic reference, an external activity marker was placed at the point of the shoulder for lateral images and on the ventral midline of the neck for ventral images. This marker was 2.5 inches long and consisted of 3 adjacent radiation sources.

5.3.3. Lymphatic dissection

An embalmed equine specimen was dissected to examine the relevant anatomic structures and the regional lymph nodes.

5.4. Results

A large radiation source was detected at the site of injection in all images obtained. On the lateral view, two lymph nodes took up a detectable amount of radiotracer within 20 minutes of injection and remained bright throughout the experiment (Figure 5.1A). These nodes were located ventral and slightly caudal to the site of injection and were cranio-dorsal to the external radiation source at the point of the shoulder (Figure 5.1B). The slight shift in location of the nodes with respect to the injection site in these two images was due to differences in the horse's head position during image acquisition. The lymph nodes were brighter at 70 min compared to 20 min, indicating progressive accumulation of ^{99m}Tc during this time. The nodes were also detectable on the ventral view, but they produced less distinct images because they were located farther from the gamma camera—which increased the scatter of the signal and amplified the

motion artifact. However, the radiotracer appeared to be concentrated in lymph nodes that were lateral to both the site of injection and the external radiation source on ventral midline (Figure 5.1C, Figure 5.1D).

An embalmed specimen was dissected to examine the relevant anatomic structures (Illustration 5.1). It was determined that the SLNs detected were in the superficial cervical lymph node cluster. These lymph nodes are located cranial to the scapula, on the cranial border of the subclavius muscle. They sit between the cleidomastoideus and omotransversarius muscles laterally and the omohyoideus muscle medially. The superficial cervical lymph nodes drain the superficial structures of the neck, thorax, and forelimb. The efferent lymphatic vessels leaving the superficial cervical lymph nodes enter the bloodstream either directly to the veins at the thoracic inlet or indirectly via the caudal deep cervical nodes.

Figure 5.1. Lymphoscintigraphy in the horse. A radiotracer was injected s.c. in the neck of a horse and images were taken using a gamma camera. Lateral images are shown from 20 min post injection with no marker (A) and 70 min post injection with a marker at the point of the shoulder (B). Ventral images are shown at 50 min post injection with a marker on ventral midline a third of the way up the neck (C) and 60 min post injection with a marker on ventral midline near the thoracic inlet (D). Inj, site of injection. N1, lymph node 1. N2, lymph node 2. M, external activity marker.

Figure 5.1. SLN detection in the horse (continued).

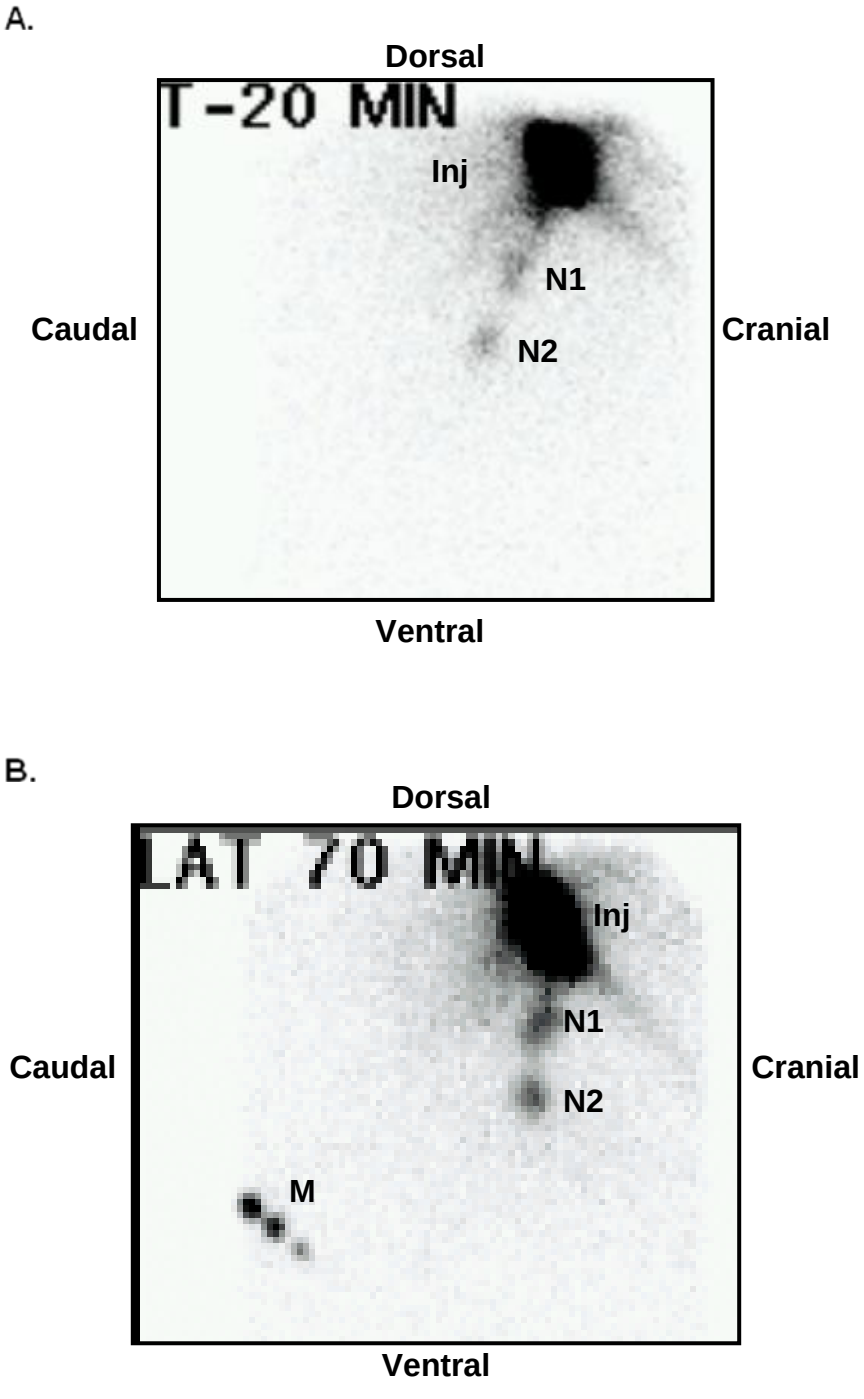
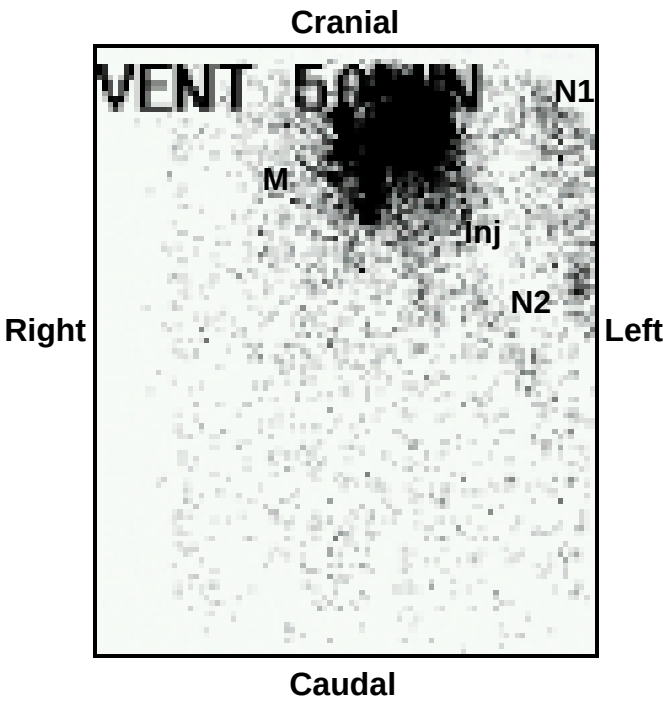


Figure 5.1. SLN detection in the horse (continued).

C.



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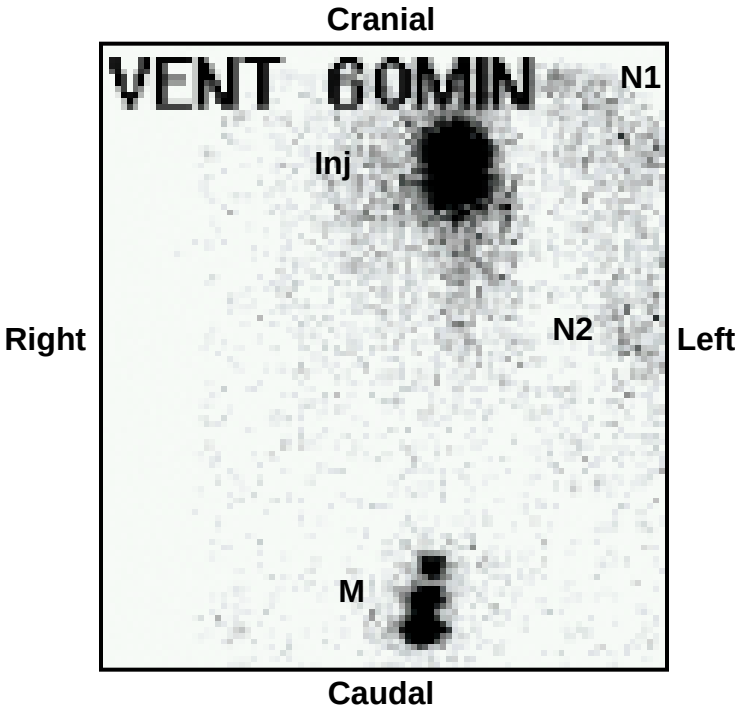


Illustration 5.1. Cadaver dissection. An embalmed miniature horse was dissected to visualize the anatomy relevant to SLN identification. (A) Illustration of relevant lymphatic anatomy. X, site of radiotracer injection. Lymph nodes represented include: 1, superficial cervical; 2, caudal deep cervical; 3, middle deep cervical; 4, cranial deep cervical; 5, lateral retropharyngeal; 6, medial retropharyngeal; 7, parotid; 8, mandibular. (B) Photograph of dissected specimen. (C) Diagram for orientation. The dissected region is indicated by a square.

Illustration 5.1. Cadaver dissection (continued).

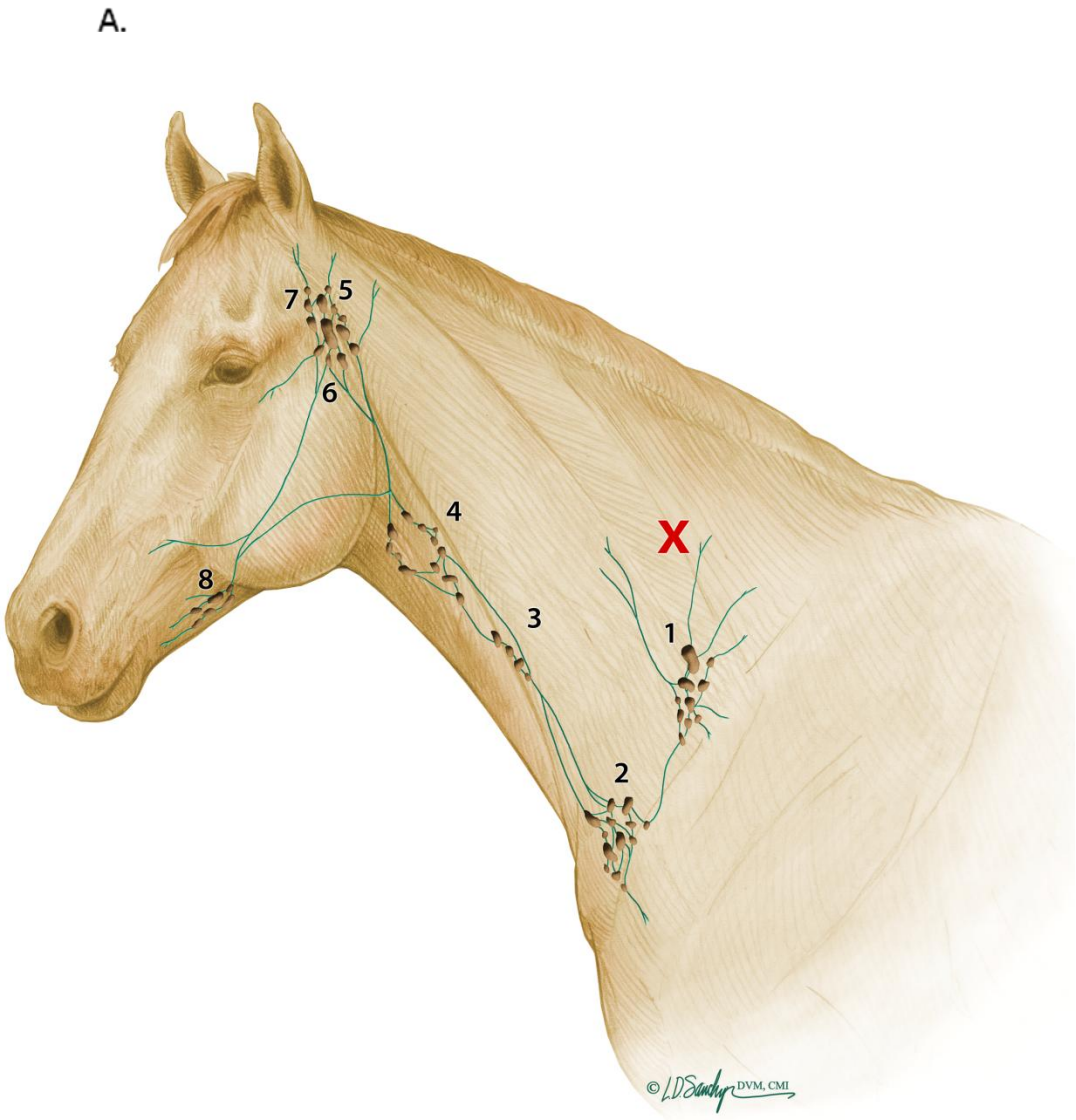
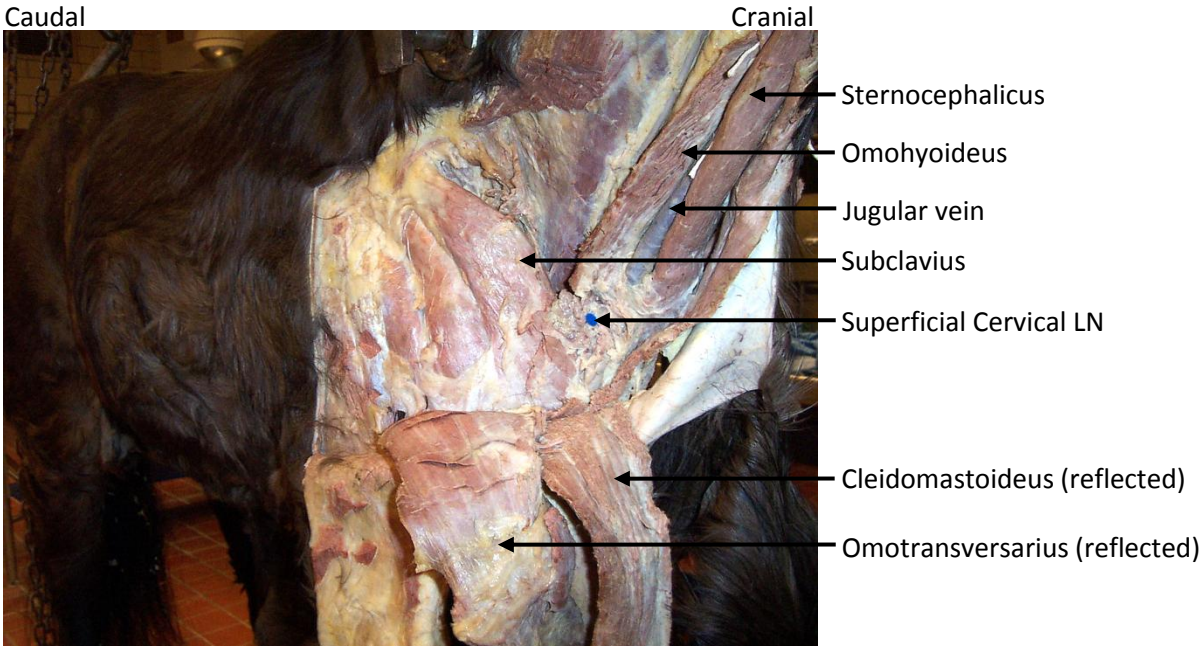
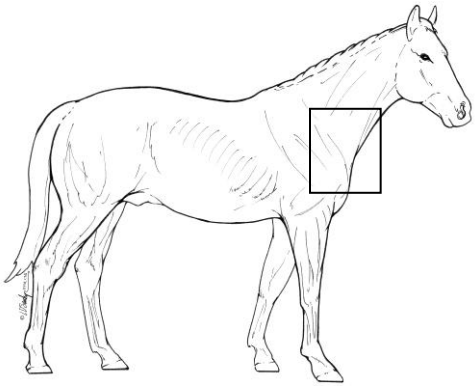


Illustration 5.1. Cadaver dissection (continued).

B.



C.



5.5. Discussion

We adapted a lymphoscintigraphic method for SLN identification in human and small animal oncology patients for use in the horse. Although horses commonly develop cutaneous neoplasms—particularly equine sarcoid, squamous cell carcinoma, and dermal melanoma—these conditions are primarily locally aggressive and treatment of metastatic disease is rarely pursued (MacGillivray et al., 2002). Therefore, we do not propose SLN identification as a therapeutic or prognostic indicator for equine oncology patients at this time. Instead, this technique will be useful in experimental settings to analyze the immune response to vaccination.

Equine vaccinations are commonly administered i.m. in the neck. However, we were unable to measure lymphatic drainage of radiotracer administered in the muscle. In contrast, the radiotracer rapidly accumulated in SLNs in the superficial cervical cluster when administered s.c. This indicates that lymphatic drainage from skeletal muscle in the horse is less efficient compared to lymphatic drainage from s.c. tissue. Interestingly, multiple studies have demonstrated that i.m. vaccinations in humans generate stronger immune responses and have a lower incidence of injection site reactions compared to s.c. vaccinations (Ruben and Jackson, 1972; Fisch et al., 1996; Mark et al., 1999; Cook et al., 2006; Ikeno et al., 2010). This creates an apparent paradox; although antigen localization to secondary lymphoid organs is required for an immune response, antigen delivered to skeletal muscle (which has poor lymphatic drainage) generates a stronger immune response. The reasons for this finding are unknown but may be due to a prolonged depot effect of antigen injected in the muscle or differences in the transport of antigen by dendritic cells from different tissues. This observation alone justifies a better understanding of vaccination sites and routes in the horse.

Local lymphatic structures serve to bring antigen, antigen-presenting cells, and lymphocytes into close proximity. This process is required for the development of a successful immune response in the SLN. Understanding this process in more detail will facilitate the study of the anti-vaccine immune response and encourage the development of improved vaccination strategies. In addition, identifying the SLN will assist analysis of the migration of antigen-loaded, monocyte-derived DCs and promote their use as cellular vaccine adjuvants.

5.6. Acknowledgements

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CHAPTER 6:

FINAL CONCLUSIONS AND DISCUSSION

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that are capable of stimulating protective immunity while maintaining tolerance to self. Therefore, DCs are essential to the development of an effective immune response (Banchereau and Steinman, 1998). Much less is known about DCs from domestic species compared to humans and laboratory animals, although these cells possess similar potential (Vecchione et al., 2002). I have endeavored in this dissertation to characterize the phenotypic and functional attributes of equine monocyte-derived DCs to promote the advancement of veterinary immunology. In particular, my objectives were twofold: (1) to validate the immunotherapeutic potential of equine DCs; and (2) to use DCs as a laboratory tool to study fundamental aspects of the equine immune system.

6.1. Validation of DC immunotherapeutic potential

A functioning adaptive immune response is critical to maintaining a state of health in which foreign pathogens are eliminated and self tissues are tolerated. Adaptive immunity contributes to many medical conditions, ranging from infectious processes to autoimmunity to cancer (Steinman. 2011). For many of these diseases, only crude treatment options exist. For example, autoimmunity results from an aberrant immune response to a specific self-antigen. However, the only treatment available for many autoimmune conditions is limited to global immune suppression that leaves the patient predisposed to secondary infections; we lack the ability to specifically suppress the autoimmune response and so we must suppress the entire immune response. In contrast, we are often unable to break tolerance to tumor-specific antigens and must rely on systemic chemotherapy to damage rapidly growing cells—both healthy and neoplastic (Vergati et al., 2010).

DCs initiate T cell activation and, depending on their maturation state, drive T cell differentiation towards a variety of effector responses, anergy, or tolerance. Therefore, DCs offer an exciting new treatment strategy to enhance, alter, or suppress the immune system in an antigen-specific manner. For example, injection of antigen-pulsed, *immature* monocyte-derived DCs induces antigen-specific inhibition of effector T cells in humans (Dhodapkar et al., 2001; Dhodapkar and Steinman, 2002). This strategy may be used to treat autoimmune diseases without the side effects associated with traditional immunosuppressive therapy. Conversely, injection of antigen-pulsed, *mature* monocyte-derived DCs induces broad antigen-specific immunity (Dhodapkar et al., 1999; Palucka et al., 2006). DCs may be used as cellular vaccine adjuvants in this manner to improve vaccine efficacy against pathogenic microorganisms or to break tolerance for cancer-specific antigens. DCs may be generated and pulsed with antigen *ex vivo,* or antigen may be targeted to specific DC subsets *in situ* (Bonifaz et al., 2004).

Dendritic cell immunotherapy may also be used to treat a variety of veterinary diseases. For example, no effective vaccines exist against equine herpesvirus-1 (EHV-1) or *Rhodococcus equi* (*R. equi*), both of which are intracellular pathogens that require strong cellular immunity (Breathnach et al., 2005; Giguere et al., 2011). DCs might be used as powerful adjuvants to produce vaccines that protect against these diseases. Similarly, DC-based vaccines could train the immune system to attack neoplasms like equine sarcoid. DCs may be used to alter the immune response and alleviate allergic conditions or inflammatory airway disease. In addition, tolerogenic DCs could treat autoimmune diseases like pemphigus foliaceus and recurrent uveitis. This project has contributed to the body of knowledge required to begin developing some of these treatments.

To validate the use of monocyte-derived DCs, I first wanted to assess whether these cells possessed typical DC characteristics that were not present in monocyte-derived macrophages. I found that the DCs were distinct from macrophages with respect to their surface marker expression, cytokine production, endocytic activity, and response to stimulation—all in a manner consistent with human DCs. Subsequent experiments revealed that DCs were much more potent in their ability to induce autologous T cell proliferation in response to both self-antigen (in the AMLR) and vaccine antigen *in vitro*. Importantly, DCs were unique in their ability to generate a primary T cell response. Therefore, equine monocyte-derived DCs possess characteristics typical of DCs from other species, including superior antigen-presenting capabilities, which are not present in equine monocyte-derived macrophages.

I also wanted to better understand the DC maturation state, since this process is so fundamental to DC biology and has profound functional consequences (Macagno et al., 2007). Measuring the maturation state of DCs had previously proven difficult due to the heterogeneity of the cell populations analyzed and the use of conflicting experimental protocols. However, I demonstrated that stimulating equine DCs with UV-inactivated *Escherichia coli* (*E. coli*) resulted in increased cell surface expression of MHC class II as well as increased expression of IL-12/IL-23p40 and IL-23p19 mRNA. Therefore, equine DCs were capable of responding to bacterial stimuli in a biologically significant manner. These results also demonstrated that equine monocyte-derived DCs consisted of a heterogeneous population of mature and immature cells. This finding was later confirmed by flow cytometric analysis of a more enriched DC population, which revealed the presence of both mature ($CD206^{low}CD86+$) and immature ($CD206^{high}CD86-$) DCs. Taken together, these findings show that equine monocyte-derived DCs possess characteristics consistent with DCs from other species that are not present in monocyte-derived

macrophages. This validates the immunotherapeutic potential of equine DCs and supports their use as a cellular vaccine adjuvant.

In particular, equine sarcoid tumors represent a unique model to evaluate the clinical use of equine monocyte-derived DCs. Equine sarcoid is the most common cutaneous neoplasm of the horse, accounting for greater than 50% of all skin cancers and composing 1 to 2% of all referred equine clinical cases (Angelos et al., 1988; Marti et al., 1993). These tumors occur exclusively in the skin (permitting easy sample collection and monitoring of response to treatment), do not metastasize to other organs, are often refractory to conventional therapies, occur naturally, and have a viral etiology (Goodrich et al., 1998). The development of equine sarcoid is associated with oncogenic bovine papillomavirus 1 and 2 (BPV-1/2). Importantly, the BPV-specific transforming E5 protein is expressed in tumor cells but not in normal skin samples from sarcoid-affected horses (Kinnunen et al., 1999; Carr et al., 2001). Therefore, E5 can serve as a tumor-specific antigen in DC-adjuvanted anti-cancer vaccines and allow DCs to promote an anti-tumor immune response.

Equine sarcoid is a clinically relevant condition in veterinary medicine. These tumors are often locally aggressive and may grow to massive dimensions, causing much discomfort for the horse and interfering with normal function. Traditional treatment options for equine sarcoids include surgical excision, cryotherapy, laser excision, nonspecific immunotherapy, interstitial brachytherapy (internal radiotherapy), hyperthermia, and chemotherapy (Goodrich et al., 1998). While some of these treatments have produced favorable results, many studies have demonstrated inconsistent responses, and no single therapy has proven universally successful (Scott and Miller, 2003). Furthermore, tumor recurrence and tumors that are refractory to

treatment are commonly encountered. In particular, horses with large tumors, multiple tumors, or rapidly growing tumors respond poorly to treatment.

BPV-induced oncogenesis in the horse and the response to DC immunotherapy will provide valuable insight into the pathogenesis and treatment of several virally-induced human neoplasms, including human papilloma virus (HPV)-induced cervical carcinoma (Moody and Laimins, 2010). The continued need for animal models of this human disease has recently been expressed (Borzacchiello et al., 2009). The sarcoid model also provides a naturally-occurring disease in an outbred, diverse population of individuals. This is a more accurate representation of the challenges inherent in developing DC cancer vaccines in humans when compared to artificially-induced cancers in inbred rodent populations. Furthermore, clinical trials can be performed at a fraction of the cost and in much less time in equine patients compared to similar studies in humans. The data presented in this dissertation will facilitate the development of DC-adjuvanted cancer vaccines in the horse and permit analysis of the resulting immune response.

Vaccines using antigen-pulsed, *ex vivo*-derived DCs as a cellular vaccine adjuvant have shown some promise in clinical trials in humans (Ueno et al., 2010). However, many of these vaccines have failed to reach the potential efficacy suggested by rodent studies (Dauer et al., 2008). One major complication with this treatment is the inefficiency by which injected DCs travel to the draining lymph node where they can activate vaccine antigen-specific T cells (Blocklet et al., 2003; Figdor et al., 2004). I have developed a lymphoscintigraphic technique for identifying the sentinel lymph node in the horse. By identifying the DC vaccine-draining lymph node with this procedure, the migratory capacity of injected DCs can be assessed. In addition, the sentinel node can be analyzed to study the interaction of *ex vivo*-derived DCs with

autologous T cells *in vivo*. Therefore, this technique will facilitate the development of DC-adjuvanted vaccines in the horse.

6.2. DCs as a laboratory tool

I developed a protocol to measure the DC-induced T cell response using multi-color flow cytometry. In addition to the functional characterization of equine DCs compared to macrophages, this system permitted the use of DCs to study fundamental immunological processes *in vitro*. These findings contribute to our knowledge of the equine immune system and demonstrate the value of DCs as a research tool.

T cells that responded to DC stimulation expressed higher levels of CD4 and MHC class II on the cell surface when compared to non-responsive cells. This finding, while unexpected, reveals an interesting aspect of the T cell response to antigen stimulation. In contrast, CD3 expression was equivalent between proliferating and non-proliferating cells.

There is only a small discussion in the literature of differences in CD4 expression among helper T cell subsets. To our knowledge, we report the first demonstration of CD4 upregulation in a polyclonal population of non-murine cells. CD4 is a TCR co-receptor that brings the tyrosine kinase Lck into close proximity with the TCR following ligand binding to initiate phosphorylation events associated with cellular activation (Weiss and Littman, 1994). CD4 expression is reported to increase following recognition of antigen by T cells (Ridgway et al., 1998; Li et al., 2007). However, it has been shown in cell lines from TCR transgenic mice that Th1 cells express more CD4 than Th2 cells (Itoh et al., 2005). Furthermore, the surface density of CD4 is inversely correlated with the avidity of the TCR ligand required for signaling to occur; cells with increased CD4 expression can respond to lower avidity ligands (Viola et al., 1997;

Lovatt et al., 2006). Therefore, several explanations exist for the increased CD4 expression detected in the DC-responsive T cells in the equine AMLR. These cells could be upregulating CD4 after activation, they could be enriched for Th1 cells, or the $CD4^{high}$ cells could be more likely to proliferate in the presence of low avidity ligands. I favor the latter explanation because the AMLR-responsive T cells are thought to be responding to self-antigens, which are generally low avidity ligands. Regardless, further research is required to answer this question definitively (Liu et al., 1995). However, it is clear that CD4 expression on equine helper T cells can vary, and this heterogeneity of CD4 expression may have important functional consequences.

In contrast to mice, MHC class II molecules are expressed on T cells of most other species, including humans and horses (Lunn et al., 1993; Holling et al., 2004). Only activated T cells are MHC class II-positive in humans and, although equine T cells also upregulate MHC class II molecules when stimulated, nearly 100% of peripheral blood T cells from adult horses are positive at the steady state (Reinherz et al., 1979; Bendali-Ahcene et al., 1997). The functional significance of MHC class II expression on T cells is not completely understood, but these non-professional APCs are thought to present self-antigen and induce anergy in autoreactive T cells (Costantino et al., 2012). The increased expression of MHC class II molecules in the DC-responsive T cells in my experiment is consistent with an activated phenotype. This is the first demonstration that equine T cells upregulate MHC class II molecules after stimulation by DCs, proving that this response is not only induced by mitogens and suggesting that this increase also occurs following T cell stimulation *in vivo*.

I have also used equine DCs to confirm that a subset of conventional equine T cells expresses FoxP3 following stimulation through the TCR. This indicates that FoxP3 expression in the horse—unlike the mouse—is regulated similarly to humans. Furthermore, the activation-

induced expression of FoxP3 by conventional T cells was associated with the production of a more immunosuppressive cytokine profile. These findings suggest that FoxP3 expressed by activated T cells *in vivo* might help modulate the developing immune response, which would represent a novel mechanism for immune regulation.

Dendritic cells were also used to characterize the population of cells that proliferate in response to vaccine antigen *in vitro*. Previous experiments measured antigen-specific lymphocyte proliferation using tritiated thymidine assays (Hammond et al., 1999). Such experiments are based on the assumption that the responder population is composed entirely of T cells. I used flow cytometry to demonstrate that this is not the case—the cells that proliferated in response to antigen-presenting DCs consisted of both T cells and plasma cells. Because plasma cells only proliferated when exposed to DCs and KLH together, they must be proliferating in response to some DC-derived signal. Such a signal could be provided directly by the DCs or indirectly by DC-activated T cells. This finding emphasizes the benefit of flow cytometry-based proliferation assays and might reveal a previously unappreciated function of DCs in immune activation.

Taken together, these results illustrate the value of using equine monocyte-derived DCs as a research tool to study the immune system *in vitro*. Analyzing the interactions between DCs and lymphocytes enables a detailed analysis of processes related to antigen presentation and initiation of the adaptive immune response. These experiments will be useful to advance our understanding of the response to vaccination and may promote the development of improved vaccines. For example, measuring antigen-specific T cell proliferation using multi-color flow cytometry as described in this dissertation could permit analysis of potential vaccine antigens *in vitro*. Optimal antigens could be identified by the enhanced ability of DCs to induce antigen-

specific T cell proliferation to these antigens, and this information could be used to inform effective vaccine design.

The influence of potential adjuvants on the nature of the vaccine-induced immune response could also be monitored using this technique by measuring the subset and cytokine profile of proliferating T cells. I measured the adjuvant effect of LPS in my experiments and found that LPS-stimulated DCs did not differ in their expression of surface markers or in their ability to induce antigen-specific T cell proliferation. LPS treatment also had no effect on the subset or cytokine profile of T cells that responded to the antigen-presenting DCs. This suggests that LPS does not possess adjuvant activity at the concentration used and may indicate that multiple stimuli are required to alter the ability of DCs to activate antigen-specific T cells. In addition, the effect of LPS may have been masked by endotoxin contamination of some experimental reagents. In contrast, exposure of monocyte-derived DCs to UV-inactivated *E. coli* promoted an increased expression of MHC class II molecules. Therefore, vaccine formulations may require multiple TLR ligands to efficiently induce maturation of monocyte-derived DCs. Understanding the ability of adjuvants to alter DC-induced T cell activation will assist the selection of appropriate adjuvants to promote the most effective immune response. For example, a Th1 immune response mediates protection against *R. equi* infection in foals. Identifying adjuvants that stimulate DCs to promote Th1 cell differentiation *in vitro* will help to identify the most promising adjuvants prior to *in vivo* trials.

In summary, I have characterized the phenotype and function of equine monocyte-derived DCs. In doing so, I have provided evidence that supports the use of these cells as an immunotherapeutic agent to treat a variety of diseases, including equine sarcoid. In addition, I have established a technique to closely analyze antigen presentation and T cell activation, and I

have used this protocol to answer questions about fundamental aspects of the equine immune system. I anticipate that the findings reported in this dissertation will promote the development of improved vaccinations and treatment strategies for equine patients.

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